# THE EFFECT OF FEEDING SUGAR UPON THE ESTERASE CONTENT OF THE BLOOD SERUM AND ORGANS IN PHOSPHORUS POISONING.

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The so called sparing action of carbohydrates upon protein metabolism has long been known. The mechanism of the process is probably closely related to the protective effect of a carbohydrate diet upon certain types of poisoning believed to be associated with increased protein metabolism. Opie and Alford[1] demonstrated that the feeding of starches and sugars in large amounts renders experimental animals (rats) less susceptible to poisoning by chloroform and phosphorus. Graham[2] showed that the animals whose livers contained large amounts of glycogen suffered less readily from delayed chloroform poisoning, even when the anesthesia was continued for considerable periods of time. A series of experiments carried out in this laboratory in 1915–16 fully confirmed these results. It seemed probable that this protective action of carbohydrates was in some way dependent upon the intracellular enzymes. This report presents the results of a study, made at that time, of the effects of feeding sugar and of poisoning by phosphorus and chloroform upon the esterase content of the blood serum and of extracts of the liver, spleen, and kidneys.

Interest in the lipolytic activity of blood serum and tissues dates from 1896 when Hanriot[3] demonstrated the presence in blood serum and in extracts of liver and pancreas of a ferment capable of hydrolyzing fats and oils. Hanriot also

[1] Opie, E. L., and Alford, L. B., *J. Am. Med. Assn.*, 1914, lxii, 895; *J. Exp. Med.*, 1915, xxi, 1, 21.

[2] Graham, E. A., *J. Exp. Med.*, 1915, xxi, 185.

[3] Hanriot, M., *Compt. rend. Acad.*, 1896, cxxiii, 753, 833.

used monobutyrin as substrate. He called the ferment "lipase." In 1900 Kastle and Loevenhart,[4] using ethyl butyrate as substrate, proved the presence of lipase (esterase) in a number of organs and tissues, notably in the liver, stomach, and small intestine. Loevenhart[5] made further studies and found this enzyme in varying amounts in all tissues tested, being present in greatest abundance in the locations in which fat synthesis is known to take place. Quinan[6] studied the ester-splitting ferments of the liver, kidney, and muscle of guinea pigs, and concluded that the enzyme concentration per gm. of tissue is characteristic of, and constant for each organ. He also suggested that the quantitative relations between the lipase (esterase) content of different organs and tissues may be proportional to their cellularity.

The esterolytic power of the blood serum in various pathologic conditions arising spontaneously and produced experimentally has been studied by a number of investigators. Whipple,[7] Jobling, Eggstein, and Petersen,[8] and Sagal[9] observed an increase in the serum esterase in the pathologic conditions associated with destruction of liver substance, such as phosphorus and chloroform poisoning.

The concentration of esterase in diseased tissues has not been so extensively studied. Winternitz and Meloy[10] found that when microscopic fat was present in the liver, the esterolytic activity was decreased. The diminution was not, however, proportional to the amount of visible fat present. The esterase content of the kidneys varied greatly in nephritis. Quinan,[11] working with guinea pigs, found that the loss of esterolytic ferment, per gm. of liver, after prolonged intoxication with chloroform may be as great as 38 per cent. Quinan suggests that chloroform disturbs the "lipase balance," because he observed a decrease in the amount of that enzyme in the liver and a corresponding increase in the kidneys and muscle. Jobling, Eggstein, and Petersen[8] found that liver tissue showing fatty degeneration, obtained from animals poisoned with phosphorus or chloroform contained a decreased amount of esterase.

Loevenhart,[5] on the other hand, states that the fatty changes occurring in phosphorus poisoning are not due to changes in the amount of esterase in the tissues, as no disturbances of this character were noted. Ducceschi and Almagia[12] found no changes in the ester-splitting power of the livers of animals poisoned

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[4] Kastle, J. H., and Loevenhart, A. S., *Am. Chem. J.*, 1900, xxiv, 491.

[5] Loevenhart, A. S., *Am. J. Physiol.*, 1902, vi, 331.

[6] Quinan, C., *J. Med. Research*, 1915, xxxii, 45.

[7] Whipple, G. H., *Bull. Johns Hopkins Hosp.*, 1913, xxiv, 357.

[8] Jobling, J. W., Eggstein, A. A., and Petersen, W., *J. Exp. Med.*, 1915, xxii, 707.

[9] Sagal, Z., *J. Med. Research*, 1916, xxxiv, 231.

[10] Winternitz, M. C., and Meloy, C. R., *J. Med. Research*, 1910, xxii, 107.

[11] Quinan, *J. Med. Research*, 1915, xxxii, 73.

[12] Ducceschi, V., and Almagia, M., *Arch. ital. biol.*, 1903, xxxix, 29.

with phosphorus. Saxl[13] did not note any increase in the esterase of the liver in phosphorus poisoning.

The methods used by different investigators to prepare their extracts of organs have varied widely. Kastle and Loevenhart[4] in a part of their work, Quinan,[9] and others have ground up weighed quantities of the organs studied, with or without the use of sand, and have allowed the ester to come into contact with the tissue fragments. Saxl has called attention to the sources of error in this method. If the reagents remain in contact for only an hour or so the amount of acid produced from the hydrolysis of the ester is too small to be measured with accuracy. If the mixture remains in the incubator for 24 hours the acid produced by autolysis will be a source of error that cannot be neglected. Winternitz and Meloy,[10] Loevenhart,[14] and others have used clear filtrates prepared in various ways.

*Technique.*

In the experiments here reported dogs were used. The animals were anesthetized with ether and exsanguinated by opening the carotid artery. As much blood as possible was permitted to flow from the severed vessel by lowering the head and gently massaging the abdomen. The liver, spleen, and kidneys were removed at once. The capsule was separated from the kidney and the fat removed from its pelvis. The whole of each organ was then ground very fine in a meat chopper.

5 gm. of the ground tissue were weighed accurately (to 0.01 gm.) in a wide mouthed bottle. To this were added 10 cc. of glycerol (weighed, not measured). The contents were thoroughly mixed, and the bottle was tightly corked and kept in a dark place at room temperature with frequent shaking for 21 days.

After extraction in glycerol for this period 5 cc. of distilled water were added to the contents of the bottle and mixed well with a stirring rod. The mixture was filtered through a thin pad of slightly moistened absorbent cotton laid on gauze. This gauze-cotton filter was then folded over and the remaining fluid pressed out. The turbid fluid thus obtained was diluted with an equal amount of distilled water and filtered through paper until clear. 1 cc. of the filtrate was, therefore, equivalent to approximately $\frac{1}{6}$ gm. of tissue. These dilu-

[13] Saxl, P., *Biochem. Z.*, 1908, xii, 343.

[14] Loevenhart, *J. Biol. Chem.*, 1906–07, ii, 427.

tions were arbitrary, but were about the minimum that would permit satisfactory filtration through paper. The same technique was employed throughout the series of experiments. The results are therefore comparable.

The esterase content of these extracts was determined as follows: To 4 cc. of distilled water in a test-tube, 1 cc. of tissue extract, 0.25 cc. of ethyl acetate, and one drop of alcoholic solution of phenolphthalein were added. Each tube was shaken until the ester was dissolved and the mixture immediately brought to the neutral point with 0.1 N sodium hydroxide solution. 1 cc. of toluene was then added and each tube shaken forty times. They were placed in the incubator for 24 hours, and then titrated with 0.1 N sodium hydroxide solution. The titrations were all made in duplicate, and those showing differences greater than 0.20 cc. of 0.1 N sodium hydroxide solution were excluded.

## EXPERIMENTAL.

As controls, extracts were made from the organs of normal dogs which had not been submitted to any experimental procedures. They were killed by exsanguination under ether anesthesia. The results are shown in Table I.

A number of dogs were submitted to prolonged anesthesia by chloroform. The animal's head was placed in a loosely fitting round test-tube basket which had been covered with gauze. An abundant supply of air was thus assured. The chloroform was allowed to fall drop by drop from a separatory funnel upon the gauze. The anesthesia was continued for at least 4 hours. An average of 200 cc. of chloroform was used for each dog. 2 days later the animals were killed by exsanguination under ether anesthesia. The organs were ground up and extracted in glycerol for 21 days as described above. The esterolytic power of the blood serum and of the organ extracts of two animals, typical of the series, is shown in Table I.

Another series of dogs was given subcutaneous injections of phosphorus in olive oil. They were divided into three groups. Group I received fatal doses of phosphorus. Group II was given large amounts of sugar by stomach tube for 3 days before, and for 1 or 2 days after the injection of the phosphorus. Group III was treated in the same

manner as Group II but the animals were allowed to live for 6 weeks and were then killed in the manner described above. The protocols of these animals follow.

## *Group I.*

*Dog Ia.*—Weight 25 pounds. 27.5 mg. of phosphorus in oil over a period of 10 days. 2 days after the last dose the animal was very ill and was killed by exsanguination under ether.

*Autopsy.*—All the tissues were markedly bile-stained. Sections of the liver showed marked fatty degeneration with necrosis.

*Dog Ib.*—Weight 22 pounds. 15 mg. of phosphorus over a period of 4 days. 3 days later animal found dead.

*Autopsy.*—The same lesions were found as in Dog Ia, but they were less marked.

*Dog Ic.*—Weight 18 pounds. 15 mg. of phosphorus in a single dose. 4 days later animal extremely ill. Exsanguinated under ether.

*Autopsy.*—Liver yellow and soft. Sections showed marked fatty degeneration and necrosis. Hemorrhage into retroperitoneal tissues and into intestinal mucosa. Blood did not clot in 24 hours. Serum bile-stained.

*Dog Id.*—Weight 20 pounds. 20 mg. of phosphorus in two equal doses 3 days apart. 2 days later animal quite ill; killed under ether.

*Autopsy.*—Liver yellow and soft. Sections showed marked fatty degeneration and necrosis. No hemorrhages.

## *Group II.*

*Dog IIa.*—Weight 14 pounds. 150 gm. of sugar daily for 3 days, then 15 mg. of phosphorus in a single dose. 150 gm. of sugar on following day. 3 days later animal ill with cough.

*Autopsy.*—Lesions of distemper. Liver yellow. Sections show much glycogen and only a small amount of fat in the liver.

*Dog IIb.*—Weight 20 pounds. 150 gm. of sugar daily for 3 days. 15 mg. of phosphorus in a single dose. 150 gm. of sugar on following day. Animal remained in excellent condition; very lively. Killed on 4th day after injection of phosphorus.

*Autopsy.*—Liver yellowish. Small hemorrhage in retroperitoneal tissues about pancreas. Blood did not clot in 24 hours. Sections showed glycogen and some fat in the liver.

*Dog IIc.*—Weight 23 pounds. Jan. 4 to 6, 1916, inclusive, 300 gm. of sugar daily. Jan. 6. 15 mg. of phosphorus. Jan. 7 and 8, 150 gm., and Jan. 9 and 10, 300 gm. of sugar daily. Jan. 9. 10 mg. of phosphorus. Animal remained in excellent condition; very lively. Jan. 11. Killed under ether.

*Autopsy.*—Liver yellowish. No hemorrhages. Sections show large amounts of glycogen and relatively little fat in the liver cells.

*Dog IId.*—Weight 21 pounds. Treatment identical with that of Dog IIc. Killed under ether.

*Autopsy.*—Liver yellowish. Some hemorrhage about the right half of the pancreas. Sections show considerable glycogen and some fatty degeneration of the liver.

### *Group III.*

*Dog IIIa.*—Weight 30 pounds. Dec. 18 to 20, 1915, inclusive, 200 gm. of sugar daily. Dec. 20. 10 mg. of phosphorus. Remained in good condition. Feb. 9, 1916. Killed under ether anesthesia.

*Dog IIIb.*—Weight 22 pounds. Treatment like that of Dog IIIa except that 20 mg. of phosphorus were injected on Dec. 20. Feb. 9, 1916. Killed under ether.

The esterase content of the blood serum and of the organs of these animals poisoned by phosphorus is shown in Table I.

A second group of control animals was fed large amounts of sugar in 40 per cent solution by stomach tube for a period of 4 to 6 days, and killed under ether anesthesia on the day following the last feeding. The livers of all of these animals were definitely yellowish in color, the degree of coloration varying roughly with the quantity of sugar fed. All showed large amounts of glycogen in the liver cells. The esterolytic activity is shown in Table I.

## DISCUSSION.

It is seen from Table I that the esterase content of the serum and livers of normal dogs is reasonably constant. The weakest liver extract required 3.20 cc., and the strongest, 3.45 cc. of 0.1 N sodium hydroxide to neutralize the acid produced. The average was 3.30 cc. The esterolytic power of the extract of the spleen was less than one-tenth that of the liver. This is not in harmony with the statement of Fiessinger and Marie[15] and of Bergel[16] that lymphocytes are especially rich in esterase. The esterase content of the kidneys of the control animals was very variable. Winternitz and Meloy[10] found marked variations in the esterase of human kidneys. The change from the normal, usually a decrease, observed by them in

[15] Fiessinger, N., and Marie, P., *Compt. rend. Soc. biol.*, 1909, lxvii, 107

[16] Bergel, S., *Münch. med. Woch.*, 1909, lvi, 64.

nephritis was not proportional to the degree of involvement of the organ. Nephritis is not uncommon in street dogs, and a number of the animals used in these experiments showed more or less severe lesions of the kidneys. This may account for the irregularity of the results. The figures obtained for the normal animals vary too greatly

TABLE I.

*Esterase Content of Blood Serum, Liver, Spleen, and Kidney Expressed in Cubic Centimeters of 0.1 N Sodium Hydroxide Required to Neutralize the Acid Produced from Ethyl Acetate.*

| Animals. | | | Blood serum. | Liver. | Spleen. | Kidney. |
|---|---|---|---|---|---|---|
| | | | *cc.* | *cc.* | *cc.* | *cc.* |
| Control 1 | | | | 3.35 | 0.25 | 2.15 |
| " 2 | | | 0.25 | 3.25 | 0.30 | 1.15 |
| " 3 | | | 0.30 | 3.45 | 0.20 | 0.60 |
| " 4 | | | 0.40 | 3.20 | 0.25 | |
| Chloroform 1 | | | 1.05 | 3.00 | 0.20 | 2.55 |
| " 2 | | | 1.95 | 3.40 | 0.35 | 2.95 |
| Phosphorus poisoning. | Group I, phosphorus only. | Ia | 0.85 | 2.50 | 0.35 | 0.85 |
| | | Ib | | 3.55 | 0.30 | 1.05 |
| | | Ic | 1.10 | 3.35 | 0.85 | 2.25 |
| | | Id | 0.50 | 3.90 | 0.35 | 2.80 |
| | Group II, phosphorus and sugar. | IIa | 1.15 | 4.40 | 0.30 | 0.80 |
| | | IIb | 0.60 | 4.15 | 0.25 | 3.75 |
| | | IIc | 1.55 | 4.30 | 0.35 | 1.40 |
| | | IId | 1.05 | 3.75 | | 2.70 |
| | Group III, recovered. | IIIa | | 3.45 | | |
| | | IIIb | | 3.80 | 0.20 | 0.50 |
| Sugar-fed 1 | | | | 4.60 | | 1.35 |
| " 2 | | | 0.35 | 4.80 | 0.30 | 1.70 |

to permit any definite conclusion as to the average esterase content of normal dog kidneys.

In the case of the animals kept under prolonged chloroform anesthesia the amount of esterase in the blood serum indicates, as shown by Whipple, a serious lesion of the liver. Upon microscopic examination this organ showed the typical central necrosis of chloroform pois-

oning. The esterase content of the liver and spleen was not materially affected. That of the kidneys was apparently increased, at least the amount of acid produced was greater than the highest amount formed by extracts of the kidneys of any of the control animals. The results obtained with extracts of the livers of these dogs thus agree with those of Loevenhart[5] and of Ducceschi and Almagia,[12] rather than with the results reported by Jobling, Eggstein, and Petersen[8] and by Quinan.[11]

Poisoning with phosphorus likewise did not materially change the esterase content of the liver. In all except one animal there was a slight increase. In Dog Ia there was an apparent diminution. Inasmuch as this was the only animal in the series that showed such a reduction, some other plausible explanation of this single instance was sought for. Loevenhart[14] found that bile salts in 0.2 to 1 per cent solution greatly inhibited the action of clear liver extracts upon aqueous solutions of ethyl acetate and ethyl butyrate. It appeared probable, therefore, that the apparent reduction in the case of Dog Ia might be due merely to an inhibition by the bile present in the extract, for it was observed that the extract of this liver was more deeply bile-stained than was that of any of the others. The following experiment was, accordingly, carried out. To 3 cc. of distilled water in a series of test-tubes there were added 1 cc. of liver extract of "Sugar-fed 2" (the strongest extract of the entire series), 1 cc. of a solution of dog bile to make a dilution as shown in Table II, and 0.25 cc. of ethyl acetate. The mixtures were made neutral with 0.1 N sodium hydroxide, shaken up with toluene, and incubated for 24 hours. They were then titrated with 0.1 N sodium hydroxide. The results are shown in Table II. A similar but slightly less effect was also evident with the extract of the kidney of the same animal (Dog Ia). Hence it appears probable that the reduction in esterase activity of the liver of Dog Ia was only an apparent one and was due to the inhibiting action of the bile present in the extract.

It is interesting to note in this connection that Jobling, Eggstein, and Petersen[8] found that the esterase content of the blood from the hepatic vein was less than that from the portal vein. They concluded from this observation that the increased esterase content of the blood serum in phosphorus poisoning does not come from the destroyed

liver cells. In view of the results recorded in Table II it is possible that the greater concentration of bile in the blood of the hepatic vein may account for the difference observed by these authors. The question whether in jaundice the bile leaves the liver by way of the lymphatics or is absorbed directly into the blood is still a matter of dispute. But the statement of Sabin[17] that lymphatics have never been demonstrated in the adult liver beyond the capsule and the connective tissue trabeculæ strongly favors the view that the bile in jaundice is absorbed directly into the blood. In that case the concentration of bile in the blood of the hepatic vein would be greater than the concentration in any other part of the circulation.

The feeding of sugar and the consequent storing of glycogen in the liver is accompanied by a pronounced increase in the esterolytic ac-

TABLE II.

*Inhibitory Effect of Bile on the Esterolytic Activity of Extract of Dog Liver.*

| Dilution of bile. | 0.1 N sodium hydroxide. |
|---|---|
| | *cc.* |
| 1:100 | 0.85 |
| 1:200 | 2.80 |
| 1:400 | 3.75 |
| 1:800 | 4.20 |
| 1:1,600 | 4.45 |
| Control (no bile). | 4.80 |

tivity of the liver. This effect is not so evident in the other organs studied, none of which normally stores glycogen. The increase in esterase is also seen in the animals which were fed sugar before and after poisoning with phosphorus. Saikowsky,[18] Rosenbaum,[19] and Rettig[20] have noted the rapid and complete disappearance of glycogen from the liver in phosphorus poisoning. There is a coincident marked increase in the amount of visible fat.

The protective action of the feeding of sugar manifested itself both clinically and histologically. The animals which were given only

[17] Sabin, F. R., *The Harvey Lectures*, 1915–16, xi, 124.

[18] Saikowsky, *Virchows Arch. path. Anat.*, 1865, xxxiv, 73.

[19] Rosenbaum, F., *Arch. exp. Path. u. Pharmakol.*, 1882, xv, 450.

[20] Rettig, H., *Arch. exp. Path. u. Pharmakol.*, 1914, lxxvi, 345.

phosphorus became exceedingly ill, and several died as a result of the poisoning. The livers of all of them showed absence of glycogen, marked increase of visible fat, and necrosis. The dogs which were given an equivalent amount of phosphorus with feeding of sugar showed little or no evidence of illness clinically and were always playful. None died as a result of the poisoning. The livers showed glycogen still present, and some visible fat, but no necrosis.

The question as to the identity of the ferment which hydrolyzes the neutral fats (lipase) and that which splits the simple esters like ethyl acetate (esterase) is not yet positively settled. But it seems probable that the relatively small amount of visible fat present in the livers of the animals which were fed on sugar before and after poisoning with phosphorus is in some way closely related to the marked increase in the esterase content of the liver noted in these experiments. It should be observed that the feeding of sugar does not prevent the increase in serum esterase in phosphorus poisoning.

Animals which have entirely recovered from phosphorus poisoning after feeding sugar, still show for some time a slight increase in the esterase content of the liver.

## SUMMARY.

1. Clear, filtered glycerol extracts of chopped liver, spleen, and kidney contain an ester-splitting ferment.

2. The esterase content of extracts of the liver and spleen of normal dogs is reasonably constant.

3. The amount of esterase in the liver does not appear to vary to any great extent from the normal, in poisoning with chloroform and phosphorus.

4. Feeding large quantities of sugar and increasing the amount of glycogen in the liver is accompanied by a marked increase in the esterase content of that organ. This increase is also evident in phosphorus-poisoned animals which have been fed large amounts of sugar.

5. The feeding of sugar does not prevent the increase in esterase in the blood serum of animals poisoned with phosphorus.

6. The esterolytic power of extracts of the kidney varies considerably in different dogs.

# THE PEPTOLYTIC POWER OF LIVER, SPLEEN, AND KIDNEYS IN POISONING BY PHOSPHORUS AND CHLOROFORM.

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In another communication it was shown that the feeding of sugar greatly increased the esterase content of the livers of both normal and phosphorus-poisoned dogs.[1] The present paper records the results of a study of the peptolytic power of the organs of the same animals used in the previously reported experiments.

Cohnheim[2] discovered in the press juice of the intestinal mucosa a ferment, which he called erepsin, capable of splitting peptone into amino-acids. Salkowski[3] appears to have been the first to demonstrate intracellular enzymes when he showed that liver and muscle underwent "autodigestion." Vernon[4] has made extensive studies of intracellular erepsin. Abderhalden and Teruuchi[5] suggested the term "ereptase" to distinguish intracellular erepsin from the extracellular ferment of the same name in the succus entericus. Vernon[6] found ereptase in the following organs, which are given in the order of the quantity present: intestinal mucous membrane (especially that of the duodenum), kidney, spleen, lung, pancreas, liver, submaxillary gland, thyroid, adrenal, heart, brain, ovary, skeletal muscle, and blood serum. Cohnheim and Pletnew[7] demonstrated that the peptolytic activity possessed by different organs and tissues is independent of the presence of traces of blood. Abderhalden[8] found peptolytic ferments in the kidney, lung, small intestine, and ovary, but little in skeletal muscle. Raubitschek,[9]

[1] Simonds, J. P., *J. Exp. Med.*, 1918, xxviii, 663.

[2] Cohnheim, O., *Z. physiol. Chem.*, 1901, xxxiii, 451; 1902, xxxv, 134.

[3] Salkowski, E., *Z. klin. Med.*, 1890, xvii, suppl., 77.

[4] Vernon, H. M., *J. Physiol.*, 1904, xxx, 330.

[5] Abderhalden, E., and Teruuchi, Y., *Z. physiol. Chem.*, 1906, xlix, 1.

[6] Vernon, *J. Physiol.*, 1905–06, xxxiii, 81.

[7] Cohnheim, O., and Pletnew, D., *Z. physiol. Chem.*, 1910, lxix, 108.

[8] Abderhalden, E., *Z. physiol. Chem.*, 1910, lxvi, 137.

[9] Raubitschek, E., *Z. exp. Path. u. Therap.*, 1907, iv, 675.

on the other hand, was unable to demonstrate the presence of ereptase in any tissue except the intestinal mucous membrane.

Vernon[10] believes that in growing animals the ereptic power of the tissues is closely related to their functional activity and functional capacity, since the tissues of embryos contain little or no ereptase. He found the tissues of non-hibernating hedgehogs more active than those of hibernating animals. Vernon found further that the ereptic activity of tissues is only moderately affected by diet. Cats fed on a mixed diet, including meat, had the most ereptase, while those fed on bread and milk had the least. Response to changes of diet was slow.

Comparatively little work has been done upon the effect of disease upon the ereptic power of the different organs and tissues. Vernon[10] found that guinea pigs of one-half their normal weight showed less than one-half the normal tissue ereptase. In man, the ereptic power of the kidneys diminished approximately in proportion to the severity of the nephritis. Colwell[11] found the tissues of patients who had died of malignant disease poorer in ereptase than those of normal persons. But Colwell and McCormac[12] observed that the tissues of cancerous mice were more strongly peptolytic than those of normal mice. This difference was accounted for by the greater emaciation and cachexia in human cancer patients. Jacque and Woodyatt,[13] and Hamburger[14] demonstrated erepsin in the gastric juice of patients with carcinoma of the stomach. They believed that this erepsin was liberated from disintegrated cancer cells. Ereptase has been demonstrated in press juice from carcinomas.[15]

Although numerous studies have been made of the autolysis of livers of animals poisoned with phosphorus and chloroform, little attention appears to have been paid to the ereptic power proper of these organs. There is good reason for believing that the autolytic changes in these livers may not be a correct index of their ereptic activity. The only two reports of studies of the ereptase of phosphorus-poisoned livers which I have been able to find in the literature are absolutely contradictory. Thus, Bergell and Lewin[16] claim that in phosphorus poisoning the ereptic ferment of the liver is destroyed. Abderhalden and Schittenhelm,[17] on the other hand, state that the press juice of the livers of dogs poisoned with phosphorus exert as great, if not greater, influence in splitting dipeptides as that of normal dogs.

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[10] Vernon, Intracellular enzymes, London, 1908, 40 ff.

[11] Colwell, H. A., *Arch. Middelsex Hosp.*, 1909, xv, 96.

[12] Colwell, H. A., and McCormac, H., *Arch. Middlesex Hosp.*, 1909, xv, 104.

[13] Jacque, J. L., and Woodyatt, R. T., *Arch. Int. Med.*, 1912, x, 560.

[14] Hamburger, W. W., *J. Am. Med. Assn.*, 1912, lix, 847.

[15] Abderhalden, E., and Medigreceanu, F., *Z. physiol. Chem.*, 1910, lxvi, 265.

[16] Bergell, P., and Lewin, K., *Z. exp. Path. u. Therap.*, 1906, iii, 425.

[17] Abderhalden, E., and Schittenhelm, A., *Z. physiol. Chem.*, 1906, xlix, 41.

*Technique.*

The technique used in the experiments reported here was suggested by the work of Vernon.[18] In a study of the ereptase content of different organs Vernon used glycerol extracts of 1 gm. of ground tissue in 2 cc. of glycerol. He used 0.25 cc. of the extract and found that the amount of enzyme present increased up to the 21st day and slowly decreased after the 25th day. He employed a colorimetric method based upon a modification of the biuret test to estimate the amount of peptone split into amino-acids. In my experiments larger amounts of tissue and glycerol and of the extract (5 gm. of tissue in 10 cc. of glycerol, weighed, not measured; and 1 cc. of diluted extract) were used in the belief that the percentage of error would be thus lessened. Instead of the colorimetric method, Sörensen's formaldehyde titration method was employed.[19]

The extracts used in these experiments are the same as those used in a study of esterase reported in another paper[1] in which the details of their preparation are given. The reaction of these glycerol extracts was always slightly acid. To neutralize 1 cc. of diluted liver extract, which was the amount employed in the tests, 0.10 to 0.12 cc. of 0.1 N sodium hydroxide was required; for 1 cc. of spleen extract, 0.07 to 0.08 cc.; and for 1 cc. of kidney extract, 0.05 to 0.06 cc.

As substrate, a 4 per cent solution of Witte's peptone, always from the same original container, was employed. The peptone was dissolved in distilled water by heat, cooled, and filtered. This slightly turbid filtrate was rendered neutral to phenolphthalein when it became quite clear. 9 cc. of the peptone solution were run into test-tubes from a burette, and 1 cc. each of extract and toluene was added. Each tube was shaken eighty times, and placed in the incubator. At the end of 24 and 48 hours and 6 days, duplicate (sometimes triplicate) tubes were shaken thoroughly and the contents filtered through paper until clear. Although clear when placed in the incubator, all the tubes, except the controls, when removed for examination showed a precipitate. 5 cc. of the filtrates were removed to Erlenmeyer flasks, diluted with 45 cc. of distilled water, and 5 cc. of

[18] Vernon, *J. Physiol.*, 1904–05, xxxii, 33.

[19] Sörensen, S. P. L., *Biochem. Z.*, 1908, vii, 45.

approximately neutral 40 per cent solution of formaldehyde and three drops of phenolphthalein solution added. After standing for a few minutes the contents of the flasks were titrated with 0.1 N sodium hydroxide. The following controls were made: (1) 1 cc. of organ extract in 9 cc. of distilled water under toluene to exclude increases in acidity due to changes in the extract itself. This was not found to occur. (2) 9 cc. of peptone solution with 1 cc. of distilled water to determine the amount of preformed amino-acids in the peptone solution. This was not found to increase upon standing in the incubator. (3) 5 cc. of the formaldehyde solution were diluted with 50 cc. of distilled water and titrated to determine the amount of unneutralized acid in the formaldehyde.

## EXPERIMENTAL.

Inasmuch as the animals made the basis of the experiments reported here are the same that were used in a study of esterase,[1] in the report of which the protocols were given in some detail, it is only necessary to make the following statements concerning them. All the animals were killed by bleeding from the carotid artery under light ether anesthesia. As controls, dogs which had not been submitted to any experimental procedure were used. A series of animals was submitted to profound chloroform anesthesia for periods of 4 or more hours and killed 2 days later. The phosphorus-poisoned dogs were divided into two groups. Group I received subcutaneous injections of phosphorus in oil. Group II was given phosphorus in approximately similar proportions but with the administration of 150 to 300 gm. of sugar in solution daily by stomach tube for 3 days previous to, and 1 or 2 days after the injections of phosphorus. The "sugar-fed" animals were dogs which received 200 to 300 gm. of sugar daily for 5 or 6 days. The liver, spleen, and kidneys of each animal were removed immediately after death and the entire organ was ground fine in a meat chopper. The results of these experiments are shown in Table I.

TABLE I.

*Ereptic Power of Glycerol Extracts of Liver, Spleen, and Kidney Expressed in Cubic Centimeters of 0.1 N Sodium Hydroxide Required to Neutralize the Amino-Acids.*

| Animals. | Preformed amino-acid in peptone. | Amino-acids due to cleavage by ereptase of extract. | | |
|---|---|---|---|---|
| | | Liver. | Spleen. | Kidney. |
| | *cc.* | *cc.* | *cc.* | *cc.* |
| Control 1 | 1.35 | 2.50 | | 4.60 |
| " 2 | 1.50 | 2.05 | 1.85 | 4.45 |
| " 3 | 1.40 | 1.75 | 2.00 | 3.70 |
| " 4 | 1.25 | 2.30 | 1.70 | 4.25 |
| " 5 | 1.25 | 2.60 | | |
| Chloroform 1 | 1.35 | 2.05 | 2.10 | 4.45 |
| " 2 | 1.30 | 2.15 | 2.20 | 4.50 |
| Phosphorus Ia | | 1.80 | 1.45 | 2.70 |
| " Ib | 0.75(?) | 1.35 | 1.80 | 3.35 |
| " Ic | 1.40 | 1.25 | 2.45 | 4.75 |
| " Id | 1.45 | 1.65 | 2.75 | 5.05 |
| Phosphorus IIa | | 2.15 | 2.75 | 4.80 |
| " IIb | 1.40 | 1.45 | 1.90 | 4.20 |
| " IIc | 1.45 | 2.20 | 2.85 | 4.65 |
| " IId | 1.45 | 2.35 | | 5.50 |
| Sugar-fed 1 | 1.45 | 1.80 | 1.90 | 3.30 |
| " 2 | 1.30 | 2.45 | 2.70 | 4.95 |
| " 3 | 1.85 | 2.55 | 3.00 | 4.45 |

## DISCUSSION.

The ereptase content of the liver, spleen, and kidneys of normal dogs is reasonably constant. The results of titrations are not so uniform as those in the study of esterase. But the formaldehyde titration method is admittedly not so easily carried out, and the end-point is not so sharp as that for the titration of acids liberated by the hydrolysis of esters. The percentage of error is therefore somewhat greater. It was only after making many titrations that sufficient experience was attained to make possible reasonably uniform

results. From Table I it appears that the ereptic power of the normal kidney is approximately double that of the normal liver; and that of the liver is, in most instances, equal to, or slightly greater than that of the spleen. The position of the spleen in Vernon's[6] list indicates that he found its ereptase content greater than that of the liver.

In a considerable series of tests it was found that the ereptic activity of extracts of liver is not materially influenced by the presence of bile in a dilution of 1:200. In this respect ereptase differs sharply from esterase.

Vernon[20] obtained the highest cleavage of peptone by ereptase in a slightly alkaline solution. This was not uniform for all organs, however, a fact which led him to conclude that there is a multiplicity of ereptases instead of a single peptolytic ferment common to all organs and tissues. In my own extracts the ereptic power showed little variation in media with reactions ranging from slightly alkaline to slightly acid. In this respect it is similar to the ereptic ferment found by Jobling and Strouse[21] in extracts of leucocytes. Table I is made up from titrations of filtrates of mixtures which were slightly acid. The substrate was neutral. The acidity of the mixture was therefore due to the minute amount of acid in 1 cc. of the diluted extract.

Chloroform poisoning does not appear to reduce materially the ereptic power of the organs studied. Poisoning by phosphorus, on the other hand, causes a considerable decrease in the ereptase content of the liver, but apparently has little effect upon that of the kidneys or spleen. Neither phosphorus nor chloroform caused any definite change in the esterolytic power of the liver. Thus there appears to be a difference in the enzymic activity after poisoning by these two substances. The histologic differences are well known. Also, Opie and Alford[22] observed that a carbohydrate diet offered a greater protection against poisoning by chloroform than against poisoning by phosphorus.

Feeding sugar in large amounts for 5 or 6 days does not increase the ereptase content of the livers of normal dogs. It does, however, appear to prevent the decrease of this ferment due to phosphorus

[20] Vernon, Intracellular enzymes, London, 1908, 15.

[21] Jobling, J. W., and Strouse, S., *J. Exp. Med.*, 1912, xvi, 269.

[22] Opie, E. L., and Alford, L. B., *J. Exp. Med.*, 1915, xxi, 1.

poisoning. In this respect also the behavior of ereptase differs from that of esterase. For it was found that the feeding of sugar increased the esterolytic power of both normal and phosphorus-poisoned dogs.

## SUMMARY.

1. Glycerol extracts of liver, spleen, and kidney contain an ereptic ferment capable of splitting peptone into amino-acids.

2. Poisoning by phosphorus appears to reduce the ereptic power of the liver, and to a less extent that of the kidneys.

3. Poisoning by chloroform appears to have no appreciable effect upon the ereptase content of the liver, spleen, or kidneys.

4. Feeding of sugar to normal animals has little or no effect upon the ereptic power of the liver, spleen, or kidneys.

5. Feeding of sugar before and after poisoning with phosphorus appears to prevent the reduction of ereptic power of the liver.

# RELATIVE IRRITANT PROPERTIES OF THE CHLORINE GROUP OF ANTISEPTICS.

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Plates 78 to 80.

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In sterilizing infected wounds with hypochlorite solution one of the most important problems has been that of avoiding excessive irritation. Dakin,[1] found that sodium hypochlorite solution could be used clinically if its concentration was not greater than 0.5 per cent and if the excess alkalinity of the solution had been reduced until there was no longer color with powdered phenolphthalein.

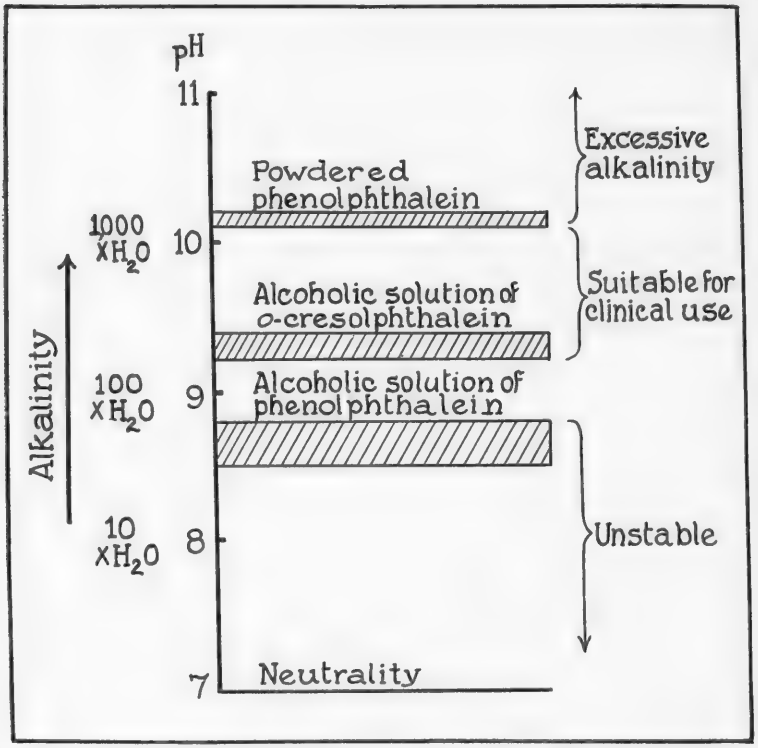
In reducing this excess alkalinity he used boric acid and thus introduced buffer salts that tend to prevent further change in the reaction of the solution. The resulting solution has come into general use as "Dakin's solution."

So many modifications of Dakin's original solution have been proposed that it seemed desirable to gain some idea of their relative irritant properties. Recent work from this laboratory[2] has emphasized the fact that Dakin's hypochlorite solution adjusted to the end-point of powdered phenolphthalein is not a neutral solution as it has often been described, but is a solution with an alkalinity of about 1,000 times that of water (pH = 10.2). Moreover, there is a lower limit of alkalinity, at about 100 times that of water (pH about 9), below which this solution becomes too unstable for clinical use. Powdered phenolphthalein alone is not a sufficient criterion of the reaction, but is the test for the upper limit of alkalinity. The end-points[2] of alcoholic phenolphthalein (pH of 8.5 to 8.8) or *o*-cresolphthalein (pH of 9.2 to 9.4) can be used as tests for the lower limit of alkalinity. It seemed desirable, therefore, in studying the relative irri-

[1] Dakin, H. D., *Brit. Med. J.*, 1915, ii, 318.

[2] Cullen, G. E., and Austin, J. H., *J. Biol. Chem.*, 1918, xxxiv, 553.

tant action of the solutions to be tested, to adjust them to these upper and lower limits of alkalinity. The relation between these limits is expressed graphically in Text-fig. 1.




TEXT-FIG. 1. Approximate alkalinity of a 0.5 per cent sodium hypochlorite solution at end-points to phenolphthalein and *o*-cresolphthalein. Also the relation between alkalinity of solutions and their availability for clinical use. Alkalinity is expressed in terms of hydrogen ion concentration (pH) and compared with that of water.

Three methods are available for the preparation of sodium hypochlorite solutions. In preparing the solution from bleaching powder, the most used and least desirable method, the calcium is precipitated from the bleach suspension by sodium carbonate and the excessive

alkalinity of the filtrate, containing sodium hydroxide (NaOH) and sodium carbonate ($Na_2CO_3$), is neutralized by boric acid, (Dakin[1]) or by sodium bicarbonate (Daufresne[3]) or by hydrochloric acid and bicarbonate (Cullen and Austin[4]).

The degree of this alkalinity is proportional to the calcium hydroxide content of the bleaching powder which is an extremely variable factor. Neutralization by either boric acid or hydrochloric acid to the end-point to powdered phenolphthalein adjusts the solution to a definite reaction (pH about 10) regardless of this variation. If, however, the same fixed quantity of bicarbonate is added to filtrates of unknown and differing alkalinity, as is the case with the Daufresne modification, the reactions of the resulting hypochlorite solutions will not be constant. Experience has shown us that this modification cannot be used with the bleaching powders of irregular composition obtainable in this country, and we have, therefore, not used it in these experiments.

Sodium hypochlorite solutions may be prepared more conveniently and economically by passing chlorine gas into a sodium carbonate solution.[2] The reaction for a definite hypochlorite concentration is determined by the strength of the carbonate solution used. A third method, that of the electrolysis of a brine solution, is also economical and convenient and is especially suitable for use on ships.[5] Alkali in some form is sometimes added to increase the stability of the solution.

There are many other hypochlorite solutions which have been more or less widely used. A calcium hypochlorite solution and a bleaching powder filtrate, which had been treated with insufficient carbonate to remove all the calcium, were used as types of solutions with low buffer action. Eusol,[6] the solution used extensively by the British Army, is made from equal parts of boric acid and bleaching powder. The boric acid in this case is added to liberate free hypochlorous acid rather than with the purpose of preparing a hypo-

[3] Daufresne, M., *Presse méd.*, 1916, xxiv, 474.

[4] Cullen and Austin, *Proc. Soc. Exp. Biol. and Med.*, 1917–18, xv, 41.

[5] Dakin, H. D., and Dunham, E. K., A handbook of antiseptics, New York, 1918, 116.

[6] Smith, J. L., Drennan, A. M., Rettie, T., and Campbell, W., *Brit. Med. J.*, 1915, ii, 129.

chlorite solution of low alkalinity. The resulting solution is, however, alkaline in reaction. The reasons for the selection of the other solutions will be self-evident.

## EXPERIMENTAL.

### *Choice of Solutions.*

We have tested Dakin's solutions made (1) by adjustment of the excessively alkaline bleaching powder-carbonate filtrate with boric acid to the definite end-points of (*a*) powdered phenolphthalein (pH of 10.2) and (*b*) 0.1 per cent alcoholic *o*-cresolphthalein (pH of 9.3); (2) by the adjustment of the same filtrate with hydrochloric acid and sodium bicarbonate to a reaction between these points (Cullen and Austin[4]); (3) by the chlorine-carbonate method to the same reactions (*a* and *b*) as in (1); and (4) by electrolysis. We have further tested (5) a weakly alkaline (nearly neutral) hypochlorite solution; (6) an excessively alkaline hypochlorite solution (dilute Javelle water); (7) sodium hypochlorite solutions containing a small amount of calcium but no carbonate; (8) a calcium hypochlorite solution; (9) a eusol solution; (10) a carbonate control with a pH of 9; and finally the organic chlorine antiseptics introduced by Dakin, (11) chloramine-T[7] and (12) dichloramine-T.[8] For convenience we will refer to the reactions in terms of the pH value. Reference to Text-fig. 1 will show the corresponding alkalinity as compared with water.

### *Preparation of Solutions.*

*Solution 1, a (Bleach-Boric pH of 10.2).*—Dakin's original solution was prepared by mixing 150 gm. of a 25 per cent bleaching powder with a liter of water and allowing this to stand several hours. 100 gm. of sodium carbonate dissolved in another liter of water were added to the bleach suspension. The mixture was shaken vigorously and filtered. The filtrate was neutralized with boric acid until a small sample failed to give color with powdered phenolphthalein, and then diluted with water to a sodium hypochlorite concentration of 0.5 per cent.

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[7] Dakin, H. D., Cohen, J. B., and Kenyon, J., *Brit. Med. J.*, 1916, i, 160.

[8] Dunham and Dakin, *Brit. Med. J.*, 1917, i, 865. Dakin, H. D., Lee, W. E., Sweet, J. E., Hendrix, B. M., and Le Conte, R. G., *J. Am. Med. Assn.*, 1917, lxix, 27.

*Solution 1, b* (*Bleach-Boric; pH of 9.3*).—The solution was prepared exactly as above except that boric acid was added until the solution gave no flash of color with *o*-cresolphthalein. This solution required about five times as much boric acid as did the one at the end-point of powdered phenolphthalein.

*Solution 2* (*Bleach-HCl-NaHCO*$_3$).—The alkaline filtrate obtained as above was neutralized to the end-point to powdered phenolphthalein with 10 per cent hydrochloric acid instead of with boric acid. Then an equal volume of 6.25 per cent sodium bicarbonate solution was added and the solution was diluted with water to 0.5 per cent sodium hypochlorite concentration. This differs from the older use of sodium bicarbonate in that the solution is first adjusted to the definite end-point of powdered phenolphthalein and then made less alkaline by the addition of an arbitrary amount of bicarbonate. The reaction of this solution is slightly more alkaline than the end-point to *o*-cresolphthalein; *i.e.*, is at a pH of about 9.5.

*Solution 3, a* (*Chlorine-Carbonate; pH of 10.2*).—The solution was prepared by running chlorine gas into a solution containing 28 gm. of sodium carbonate per liter until it just failed to give color with powdered phenolphthalein. The solution was then diluted with water to 0.5 per cent sodium hypochlorite concentration.

*Solution 3, b* (*Chlorine-Carbonate; pH of 9.3*).—The solution was prepared by passing chlorine gas into a solution of 1.4 per cent sodium carbonate to a concentration of 0.5 per cent sodium hypochlorite. This was the routine procedure[9] used in The Rockefeller Institute and this solution was therefore used as a control in later experiments.

*Solution 4* (*Electrolytic*).—The solution was prepared from a 6 per cent sodium chloride solution by the passage of an electric current. The solution gave no color with powdered phenolphthalein, but gave a flash of color with both alcoholic phenolphthalein and *o*-cresolphthalein solution.

*Solution 5* (*pH Less than 8.5; Nearly Neutral*).—The solution was prepared by passing chlorine gas into a carbonate solution until it no longer gave a flash of color with alcoholic phenolphthalein. This solution having a pH of less than 8.5 was then diluted to 0.5 per cent sodium hypochlorite concentration. Since this solution contains a large proportion of hypochlorous acid and is very unstable, it was prepared immediately before each experiment.

*Solution 6* (*Dilute Javelle Water*).—The solution was made from the strongly alkaline filtrate used in preparing the bleach Dakin's by dilution to 0.5 per cent sodium hypochlorite concentration without any neutralization.

*Solution 7*.—A sodium hypochlorite solution containing no carbonate, but traces of calcium, was prepared by precipitating 95 per cent (determined experimentally) of the calcium of a suspension of bleaching powder with sodium carbonate. The solution was then diluted to 0.5 per cent sodium hypochlorite.

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[9] A 1.5 per cent sodium carbonate solution is now used.

*Solution 8.*—Calcium hypochlorite was prepared by filtering a suspension of bleaching powder. The solution was then diluted to a 0.5 per cent sodium hypochlorite equivalent.

*Solution 9.*—Eusol solution was prepared by mixing 25 gm. of boric acid with 25 gm. of bleaching powder (of about 25 per cent available chlorine) in 1 liter of water. The mixture was allowed to stand over night, filtered, and diluted to 0.5 per cent sodium hypochlorite equivalent. At first there was a definite evolution of gas, but this had ceased before the solution was used. This solution is about twice the concentration of that ordinarily used as eusol.

*Solution 10.*—An alkaline carbonate control with a pH of about 9 was prepared by adding 1 gm. of sodium carbonate and 13 gm. of sodium bicarbonate to a liter of water.

*Solution 11.*—Chloramine-T was used in 2 per cent aqueous solution.

*Solution 12.*—Dichloramine-T was dissolved to a concentration of 5 per cent in chlorcosane.[10]

### *Testing of Irritation.*

The ears of rabbits were used because of the ease with which large surfaces could be exposed to the action of the antiseptic solutions without the irritation of contiguous skin areas. The rabbits were placed on boards with part of the head projecting so that the ears hung over. Two 250 cc. glass tumblers filled with the test solutions were then so placed that each ear was immersed in a separate solution. One ear was placed in the control solution, the other in the solution to be tested.

Single exposures were not sufficient to give definite differences and the experiments were therefore run for either a 20, 40, or 60 minute period per day for several consecutive days. A 60 minute period per day somewhat approximates the length of time that patients under the Carrel[11] method are subjected to the action of hypochlorite solution, for, assuming that the solution maintains effective strength for about 5 minutes at each 2 hour instillation, we would have a total of 60 minutes per day.

Fresh solutions of known concentration were used each day and at the end of each exposure the solutions in the tumblers were

[10] Dakin and Dunham, *Brit. Med. J.*, 1918, i, 51.

[11] Carrel, A., Dakin, Daufresne, Dehelly, and Dumas, *Presse méd.*, 1915, xxiii, 397. Carrel, A., and Dehelly, G., The treatment of infected wounds, New York, 1917.

titrated and the percentage loss of hypochlorite (or hypochlorite equivalent) was recorded. Unless otherwise stated the initial concentration was 0.5 per cent sodium hypochlorite or its equivalent.

The ears were inspected before and after every exposure and daily for 10 days following the last exposure.

*Experiment 1.*—Nine white rabbits of approximately the same weight were placed in position. The left ear of each was suspended in tap water and the right in the solutions indicated. Each ear was exposed to the same solution 20 minutes daily for 7 successive days. The results are given in Table I.

TABLE I.

*Time of Exposure 20 Minutes; Number of Days 7.*

| Rabbit No. | Right ear. | | | Left ear. | |
|---|---|---|---|---|---|
| | Solution. | Irritation.* | Average fall in concentration. | Solution. | Irritation. |
| | | | *per cent* | | |
| 1 | 0.5% sodium hypochlorite (chlorine-carbonate; pH of 9.3). | +++ | 11 | Water. | 0 |
| 2 | 0.5% sodium hypochlorite (bleach-HCl-$NaHCO_3$). | +++ | 21 | " | 0 |
| 3 | 0.5% sodium hypochlorite (bleach-boric; pH of 10.2). | ++ | 9 | " | 0 |
| 4 | 0.5% sodium hypochlorite (chlorine-carbonate; pH less than 8.5). | ++++ | 19 | " | 0 |
| 5 | 0.5% sodium hypochlorite (with calcium). | + | 5 | " | 0 |
| 6 | 0.5% sodium hypochlorite (strongly alkaline). | ++++ | 11 | " | 0 |
| 7 | Alkaline carbonate control (pH of 9). | 0 | | " | 0 |
| 8 | 2% chloramine-T. | 0 | 1 | " | 0 |
| 9 | 5% dichloramine-T in chlorcosane. | ++† | 0 | Chlorcosane. | ++† |

*In the tables the degree of irritation is indicated as follows:

0, no skin irritation.

+, erosion of hair, slight edema, few petechiæ.

+ +, erosion of hair, considerable edema, more petechiæ.

+ + +, erosion of hair, marked edema, confluent petechiæ.

+ + + +, erosion of hair, very marked edema, confluent petechiæ, and superficial ulceration.

†See discussion.

The solution with a pH of less than 8.5 and the strongly alkaline hypochlorite solutions were the most irritating. The former caused slightly more irritation than the latter. At the end of the experiment the ears were swollen to twice their normal thickness, due to edema, and there were superficial ulceration and confluent petechiæ that discolored the entire surface. The hair had entirely disappeared. The solutions, bleach-boric pH of 10.2, chlorine-carbonate pH of 9.3, and bleach-HCl-$NaHCO_3$, all caused an irritation which was uniformly much less than that of the most irritating group. The bleach-boric pH of 10.2 solution seemed to be slightly less irritating than the others. The ears were edematous, the hair was completely eroded, and subcutaneous petechiæ were rather prominent. The solution containing calcium, but no carbonate, was the least irritating of the hypochlorite solutions. The hair was eroded, there was scarcely any edema, and the few petechiæ present occurred only on the proximal portion of the convexity of the ear. There was no ulceration. The alkaline carbonate control caused no irritation.

Chloramine-T in 2 per cent solution was not irritating. Dichloramine-T, 5 per cent in chlorcosane, produced an effect that differed somewhat from that caused by the other solutions. The congestion was striking; every vessel, large and small alike, seemed to be engorged with blood. There was some thickening of the ear due to a mild grade of edema. There were no hemorrhages and the skin and the hair were not eroded. Chlorcosane alone produced a similar effect but the edema was slightly less marked.

*Experiment 2.*—This was similar to Experiment 1, save that the exposures were of 40 minutes duration daily for 3 consecutive days. The results (Table II) were identical.

It appeared from Experiments 1 and 2 that the original boric acid Dakin's solution adjusted to an alkalinity of 10.2 was perhaps somewhat less irritating than the solution prepared either from bleaching powder or from chlorine and neutralized to the less alkaline end-point. As this difference was not clear-cut a third experiment was planned to eliminate any individual variations in irritability.

TABLE II.

*Time of Exposure 40 Minutes; Number of Days 3.*

| Rabbit No. | Right ear. | | | Left ear. | |
|---|---|---|---|---|---|
| | Solution. | Irritation. | Average fall in concentration. | Solution. | Irritation. |
| | | | *per cent* | | |
| 10 | 0.5% sodium hypochlorite (chlorine-carbonate; pH of 9.3). | +++ | 14 | Water. | 0 |
| 11 | 0.5% sodium hypochlorite (bleach-HCl-$NaHCO_3$). | +++ | 22 | " | 0 |
| 12 | 0.5% sodium hypochlorite (bleach-boric; pH of 10.2). | ++ | 27 | " | 0 |
| 13 | 0.5% sodium hypochlorite (chlorine-carbonate; pH less than 8.5). | ++++ | 30 | " | 0 |
| 14 | 0.5% sodium hypochlorite (with calcium). | + | 11 | " | 0 |
| 15 | 0.5% sodium hypochlorite (strongly alkaline). | ++++ | 19 | " | 0 |
| 16 | Alkaline carbonate (control; pH of 9). | 0 | | " | 0 |
| 17 | 2% chloramine-T. | 0 | | " | 0 |
| 18 | 5% dichloramine-T in chlorcosane. | ++* | 0 | Chlorcosane. | ++* |

*See discussion of Experiment 1.

*Experiment 3.*—The left ears of four rabbits were suspended in bleach-boric solution (pH of 10.2) and the right ears in the routine hospital solution (chlorine-carbonate, pH of 9.3). The ears were exposed 40 minutes daily for 5 consecutive days. They were carefully inspected before and after each exposure to the solutions and daily for 10 days subsequent to the last exposure (Table III).

Rabbit 19 was found dead on the 3rd day. The cause of death was unknown. The skin reactions observed in these rabbits were similar to those observed in Experiments 1 and 2. The hair was eroded from the skin, there were a rather severe grade of edema, congestion, and considerable subcutaneous hemorrhage without, however, any ulceration. At no time during the entire period of observation could any difference be detected between the two ears in any of the rabbits and it was therefore concluded that there was no difference in the irritant actions of these two solutions.

TABLE III.

*Time of Exposure 40 Minutes; Number of Days 5.*

| Rabbit No. | Right ear. | | | Left ear. | | |
|---|---|---|---|---|---|---|
| | Solution. | Irritation. | Average fall in concentration. | Solution. | Irritation. | Average fall in concentration. |
| | | | *per cent* | | | *per cent* |
| 19* | 0.5% sodium hypochlorite (chlorine-carbonate; pH of 9.3). | + | 32 | 0.5% sodium hypochlorite (bleach-boric; pH of 10.2). | + | 28 |
| 20 | " " | ++ | 22 | " " | ++ | 15 |
| 21 | " " | ++ | 26 | " " | ++ | 24 |
| 22 | " " | ++ | 33 | " " | ++ | 21 |

* Found dead on 3rd day.

*Experiment 4.*—Since there was some variation between individual rabbits in Experiment 3, it seemed desirable to test the other solutions with several animals, using on the control ear a hypochlorite solution rather than water. The solution prepared from chlorine and carbonate at a pH of 9.3 was taken as the control, as in Experiment 3, and the following solutions were tested: the chlorine-carbonate, pH of 10.2, and the bleach-boric, pH of 9.3 (Table IV).

TABLE IV.

*Time of Exposure 40 Minutes; Number of Days 5.*

| Rabbit No. | Right ear. | | | Left ear. | | |
|---|---|---|---|---|---|---|
| | Solution. | Irritation. | Average fall in concentration. | Solution. | Irritation. | Average fall in concentration. |
| | | | *per cent* | | | *per cent* |
| 23 | 0.5 per cent sodium hypochlorite (chlorine-carbonate; pH of 9.3). | ++ | 44 | 0.5 per cent sodium hypochlorite (chlorine-carbonate; pH of 10.2). | ++ | 43 |
| 24 | " " | +++ | 40 | " " | +++ | 48 |
| 25 | " " | +++ | 37 | " " | +++ | 29 |
| 26 | " " | +++ | 42 | " " | +++ | 43 |
| 27 | " " | +++ | 40 | 0.5 per cent sodium hypochlorite (bleach-boric; pH of 9.3). | +++ | 37 |
| 28 | " " | +++ | 32 | " " | +++ | 29 |
| 29 | " " | +++ | 47 | " " | +++ | 50 |
| 30 | " " | ++ | 25 | " " | ++ | 32 |

The results show that although individual rabbits may vary slightly in irritability, there is not sufficient difference in the irritant action of the various solutions to be detected by experiments of this nature. The differences are probably not of sufficient consequence to warrant more delicate tests.

*Experiment 5.*—Results from Experiments 1 and 2 indicate that the sodium hypochlorite solutions containing calcium, but no carbonate or borate buffers, have less irritant action than ordinary buffered solutions. In order to test this with the elimination of the individual variation in irritation shown by different rabbits, four rabbits were subjected to the action of such a solution with the same technique as in Experiments 3 and 4. The results are given in Table V.

TABLE V.

*Time of Exposure 40 Minutes; Number of Days 6.*

| Rabbit No. | Right ear. | | Left ear. | |
|---|---|---|---|---|
| | Solution. | Irritation. | Solution. | Irritation. | Average fall in concentration. |
| | | | | | *per cent* |
| 31* | 0.5 per cent sodium hypochlorite (chlorine-carbonate; pH of 9.3). | +++ | 25 | 0.5 per cent sodium hypochlorite with calcium. | +++ | 16 |
| 32 | " " | +++ | 23 | " " | ++ | 14 |
| 33 | " " | +++ | 36 | " " | + | 21 |
| 34 | " " | +++ | 35 | " " | + | 15 |

* Died after 5 days.

There is no doubt that these solutions are definitely less irritating than the ordinary Dakin's solutions; however, such solutions should not be substituted for the regular Dakin's solutions without further clinical evidence involving the solvent action on necrotic tissue, pus, effect on rate of cicatrization, etc.

*Experiment 6.*—The irritant action of electrolytically prepared hypochlorite was compared with that of chlorine-carbonate solution of the same concentration exactly as in Experiments 3 to 5, except that three 60 minute periods were used. As is indicated by Table VI, no difference between the two solutions could be detected.

TABLE VI.

*Time of Exposure 60 Minutes; Number of Days 3.*

| Rabbit No. | Right ear. | | Left ear. | |
|---|---|---|---|---|
| | Solution. | Irritation. | Solution. | Irritation. |
| 35 | 0.5 per cent sodium hypochlorite (chlorine-carbonate; pH of 9.3). | +++ | 0.5 per cent sodium hypochlorite (electrolytic). | ++++ |
| 36 | " " | +++ | " " | +++ |
| 37 | " " | ++++ | " " | +++ |
| 38 | " " | ++++ | " " | ++++ |
| 39 | " " | ++++ | " " | +++ |
| 40 | " " | ++++ | " " | +++ |
| 41 | " " | + | " " | + |
| 42 | " " | ++ | " " | ++ |

*Experiment 7.*—The irritant action of eusol, the hypochlorite solution proposed for clinical use by Smith and his colleagues,[6] was compared with that of Dakin's solution in exactly the same manner as in the preceding experiment. It should be noted that the solution is used in a 0.5 per cent sodium hypochlorite concentration rather than about 0.27 as ordinarily used.

TABLE VII.

*Time of Exposure 40 Minutes; Number of Days 5.*

| Rabbit No. | Right ear. | | | Left ear. | | |
|---|---|---|---|---|---|---|
| | Solution. | Irritation. | Average fall in concentration. | Solution. | Irritation. | Average fall in concentration. |
| | | | *per cent* | | | *per cent* |
| 43 | 0.5 per cent sodium hypochlorite (chlorine-carbonate; pH of 9.3). | ++ | 21 | 0.5 per cent sodium hypochlorite (eusol). | + | 17 |
| 44 | " " | +++ | 24 | " " | ++ | 22 |
| 45 | " " | +++ | 19 | " " | ++ | 16 |
| 46 | " " | +++ | 37 | " " | ++ | 24 |

As will be seen from Table VII, eusol is somewhat less irritating than Dakin's solution. However, in this series of experiments the ears subjected to eusol were affected more for the first 2 days than were those subjected to the Dakin's solution. After 5 days the ears in the Dakin's solution showed slightly more irritation than those in eusol.

## DISCUSSION.

The above experiments would indicate that the ears of rabbits are a satisfactory means of studying the irritating effect of solutions on normal skin. In using them, however, to show relative irritation, one should employ an irritating solution as the control, since the rabbits show considerable individual variation in their response to irritation. About one out of each four will develop two or three times more inflammation than the other three. The results may be considered from several view-points, as follows:

*Pathology.*—Irritation of the ears of the rabbits was evidenced by certain inflammatory changes. Chloramine-T, 2 per cent (Fig. 1), and the alkaline control solution produced no inflammation. No differences could be detected between the ears suspended in these solutions and those in water. Dichloramine-T, 5 per cent in chlorcosane, and chlorcosane alone caused slight edema and marked congestion. The hypochlorite solution containing calcium, but no buffer salts, caused erosion of the hair, slight edema, and a few discrete petechiæ on the proximal portion of the dorsal surface of the ears.

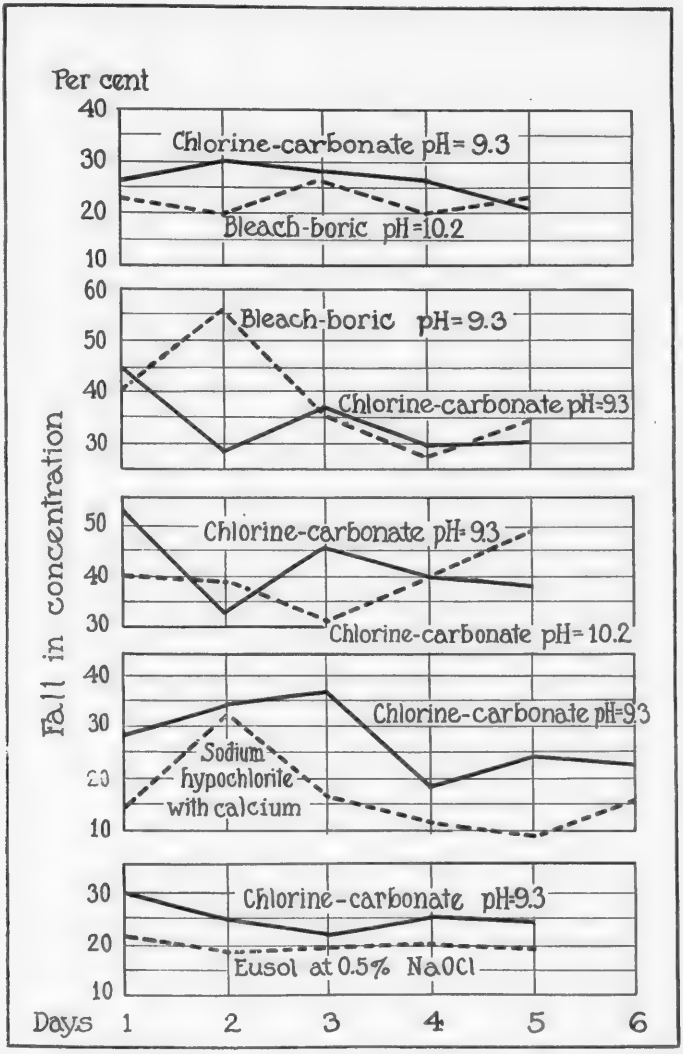
All the Dakin's solutions brought about erosion of the hair, a marked grade of edema, and confluent subcutaneous petechiæ (Fig. 2). The nearly neutral and strongly alkaline hypochlorite solutions eroded the hair, produced a very marked degree of edema, and a discoloration of the entire surface of the skin. There were superficial ulcerations (Fig. 3). Immediately after removal from the hypochlorite solutions the ear surfaces appeared clean and bleached. After a short interval congestion occurred. Between treatments there was a roughing of the surface, probably due to the serofibrinous exudate noted below in connection with the histology. After the first treatment discoloration of the ears occurred, due to petechiæ. After the last exposure a crusting of the exudate took place which fell off after a few days. Gradually the edema disappeared, the subcutaneous hemorrhages were absorbed, and 2 or 3 weeks after treatment new hair began to make its appearance. After another week the ears appeared normal.

*Microscopic Examination.*—Sections were taken from the ears after the last treatment and stained with methylene blue and eosin, as seen in Figs. 4 and 5.

Chloramine-T produced no demonstrable change (Fig. 4). 5 per cent dichloramine-T in chlorcosane and chlorcosane alone produced very marked congestion and some widening of the tissue spaces (edema). The sodium hypochlorite solution containing calcium also produced a mild degree of edema and brought about erosion of the cornified epithelium and hair and a few subcutaneous hemorrhages. There was an infiltration of small round cells into the subcutaneous tissues and a thin surface exudate, serofibrinous in character. All the Dakin's solutions caused dilatation of the small blood vessels, a rather marked grade of edema, and erosion of the cornified layer of the skin and of the hair shafts which extended down to the hair follicles themselves. There were, in addition, hemorrhages into the subcutaneous tissues, considerable infiltration of the corium with small round cells, and a thickening of the epithelium of the skin and of the hair follicles. Mitotic figures were abundant. There was evidence of fibroblastic proliferation. A thick, shaggy serofibrinous exudate was present over the surface (Fig. 5).

The strongly alkaline and nearly neutral hypochlorite solutions produced changes which were of the same type as those caused by the Dakin's solutions but greater. There were, in addition, superficial ulceration and extensive infiltration of the subcutaneous tissues with polymorphonuclear leucocytes.

*Rate of Decomposition of Solutions.*—It is, of course, well recognized that the chlorine antiseptics are decomposed by tissue material. To determine whether there was any relation between the extent of irritation and the rate of deterioration of the antiseptic, the solutions in Experiments 3, 4, 5, and 7 were titrated after each experiment. The results were analyzed in several ways. First, the percentage fall in concentration per day for each solution was studied and plotted as curves. There were so many irregularities in these curves that the composite averages for each set of four ears were compared. The composite daily curve of the four ears in each set subjected to the chlorine-carbonate control solution may be compared with the composites of the four ears in the other solutions. The curve plotted from such averages for Experiments 3, 4, 5, and 7 are given in Text-fig. 2. It would seem that while the irregularities in the curves are marked, there is a definite tendency for the solutions that were the




TEXT-FIG. 2. Curves showing fall in concentration of hypochlorite solutions in contact with ears of rabbits. Each curve represents the composite of the results obtained with the four rabbits used in each experiment. The fall in concentration is expressed as per cent of the initial concentration.

least irritating, to show a lower percentage loss. This is, of course, to be expected, for the greater the irritant action, the more marked are

TABLE VIII.

*All Solutions 0.5 Per Cent Sodium Hypochlorite or Its Equivalent.*

| Experiment No. | Rabbit No. | Right ear. Solution. | Right ear. Period average fall for each animal. | Right ear. Period average fall for set of animals. | Left ear. Solution. | Left ear. Period average fall for each animal. | Left ear. Period average fall for set of animals. |
|---|---|---|---|---|---|---|---|
| | | | *per cent* | *per cent* | | *per cent* | *per cent* |
| 3 | 19 | Chlorine-carbonate; pH of 9.3. | 32 | | Bleach-boric; pH of 10.2. | 28 | |
| 3 | 20 | " " | 22 | | " " | 15 | |
| 3 | 21 | " " | 26 | | " " | 24 | |
| 3 | 22 | " " | 33 | 28 | " " | 21 | 22 |
| 4 | 23 | Chlorine-carbonate; pH of 9.3. | 44 | | Chlorine-carbonate; pH of 10.2. | 43 | |
| 4 | 24 | " " | 40 | | " " | 48 | |
| 4 | 25 | " " | 37 | | " " | 29 | |
| 4 | 26 | " " | 42 | 41 | " " | 43 | 41 |
| 4 | 27 | Chlorine-carbonate; pH of 9.3. | 40 | | Bleach-boric; pH of 9.3. | 37 | |
| 4 | 28 | " " | 32 | | " " | 29 | |
| 4 | 29 | " " | 47 | | " " | 50 | |
| 4 | 30 | " " | 25 | 36 | " " | 32 | 37 |
| 5 | 31 | Chlorine-carbonate; pH of 9.3. | 25 | | Sodium hypochlorite and calcium. | 16 | |
| 5 | 32 | " " | 23 | | " " | 14 | |
| 5 | 33 | " " | 36 | | " " | 21 | |
| 5 | 34 | " " | 35 | 30 | " " | 15 | 17 |
| 7 | 43 | Chlorine-carbonate; pH of 9.3. | 21 | | Eusol. | 17 | |
| 7 | 44 | " " | 24 | | " | 22 | |
| 7 | 45 | " " | 19 | | " | 16 | |
| 7 | 46 | " " | 37 | 25 | " | 24 | 20 |
| Average for 20 rabbits..... | | | | 32 | | | 28 |

the roughening of the skin surface and consequent increase in area. The serous exudate and bits of tissue from the irritated surfaces would also react with and decompose the solutions.

Secondly, the results for each ear over the entire period were then compared. The average percentage loss over the entire period, *i.e.* 3 to 6 days for each ear, are given with each experiment as the "period average fall" and summarized in Table VIII. It will be seen that the average fall of the solution used most, chlorine-carbonate (pH of 9.3), for the entire twenty ears is 32 per cent and that the average fall for the twelve ears subjected to the other Dakin's solutions is 33 per cent. It should be recognized that changes in temperature, season, etc., may cause considerable variation on different days. It is, however, striking that in the solutions which cause similar biological changes, the average losses are almost identical.

## CONCLUSIONS.

From the above observations we must conclude that Dakin's hypochlorite solutions, of which the alkalinity is kept within the range of from 100 to 1,000 times the alkalinity of water (pH of 9.3 to 10.2) by means of buffer salts, have practically the same degree of irritant action. Moreover, the manner in which these solutions are prepared, whether from bleaching powder and sodium carbonate, or from chlorine and sodium carbonate, and the type of buffer salt used, either carbonate or borate, have no influence on the degree of irritation. Solutions, however, that have an alkalinity less than that indicated by the end-point of alcoholic solution of phenolphthalein (pH of 8.5 to 8.8), or greater than that indicated by the end-point to powdered phenolphthalein (pH of 10.2), are intensely irritating. The sodium hypochlorite solution from which most of the calcium has been precipitated, and calcium hypochlorite solution seem less irritating than Dakin's solutions. The alkalinity in these unbuffered solutions is due mainly to the hydrolysis of the hypochlorite and is decreased as the hypochlorite is decomposed. This, rather than any specific action of the calcium, is probably the explanation of their lessened irritant action.

It is also evident that one may expect the hypochlorite solutions to maintain effective antiseptic concentration for about the same period of time, regardless of the manner in which they are prepared.

SUMMARY.

1. The use of the ears of rabbits is proposed in testing the irritant effect of antiseptics.

2. It is necessary, because of individual variations, to use solutions having definite irritant actions as controls.

3. 0.5 per cent sodium hypochlorite solutions have minimum irritant effects over a range of alkalinity of from about 100 to 1,000 times that of water (pH about 9 to 10). Solutions may be adjusted within these limits by use of the end-points of powdered phenolphthalein, of alcoholic solutions of either *o*-cresolphthalein or phenolphthalein.

4. Sodium hypochlorite solutions kept within the above range of alkalinity by either borate or carbonate buffer salts, *i.e.* Dakin's solution, show the same irritative properties whether made from bleaching powder and sodium carbonate, or from chlorine and sodium carbonate.

5. Electrolytically prepared solutions of the same concentration have similar irritant action.

6. Solutions, however, that have an alkalinity less than that indicated by the end-point of alcoholic phenolphthalein solutions (pH of 8.5 to 8.8) or greater than that indicated by the end-point to powdered phenolphthalein (pH of 10.2) are intensely irritating.

7. 0.5 per cent sodium hypochlorite solution from which most of the calcium has been precipitated and calcium hypochlorite solution of equivalent hypochlorite concentration are only slightly irritating.

8. 2 per cent chloramine-T solution has no irritant action.

9. 5 per cent dichloramine-T in chlorcosane and chlorcosane alone irritate rabbit ears to a slight degree only.

## EXPLANATION OF PLATES.

### PLATE 78.

FIG. 1. Rabbit 8, Experiment 1. Right ear suspended 20 minutes daily for 7 days in 2 per cent chloramine-T solution. Left ear suspended for same time in water.

FIG. 2. Rabbit 2, Experiment 1. Right ear suspended 20 minutes daily for 7 days in 0.5 per cent sodium hypochlorite (bleach-HCl-$NaHCO_3$). Left ear suspended for same time in water.

PLATE 79.

FIG. 3. Rabbit 4, Experiment 1. Right ear suspended 20 minutes daily for 7 days in 0.5 per cent sodium hypochlorite (chlorine-carbonate; pH less than 8.5). This figure shows the effect of hypochlorous acid. Left ear suspended for same time in water.

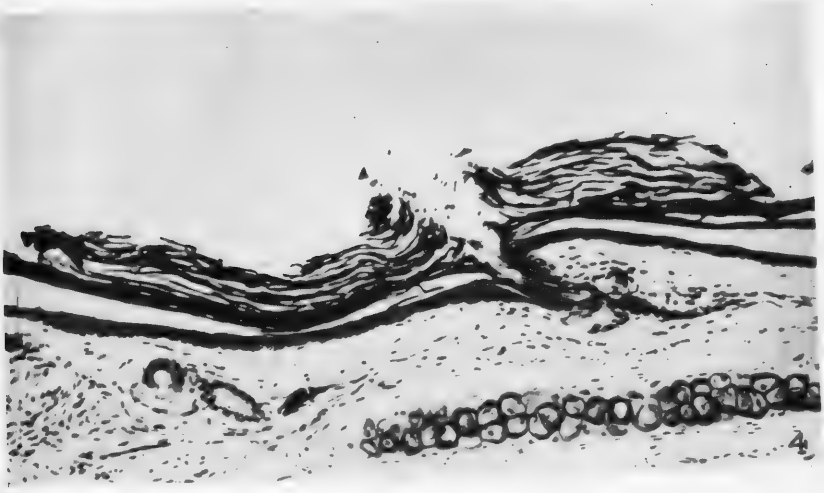
PLATE 80.

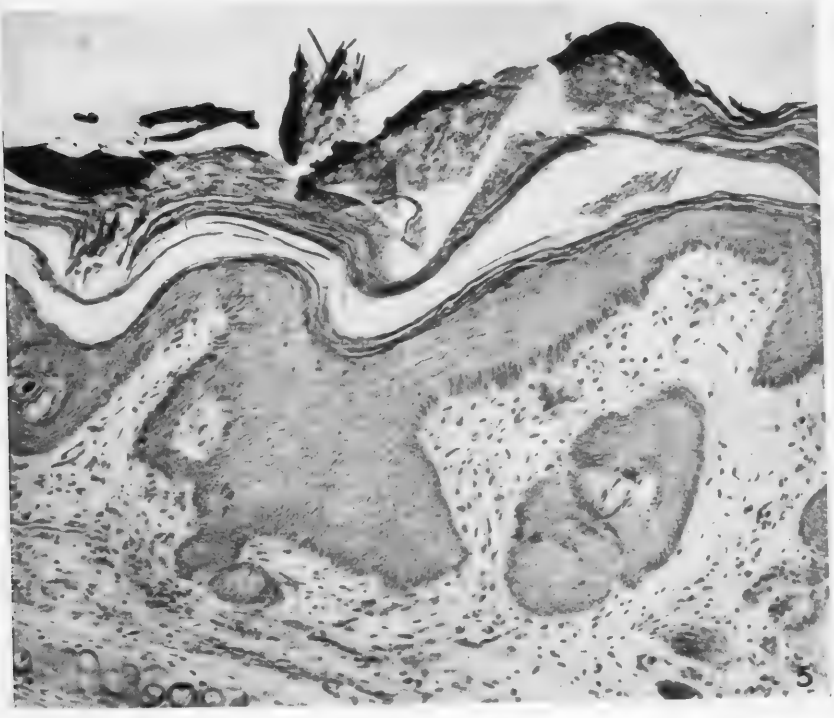
FIG. 4. Section of the skin of the ear of Rabbit 8, Experiment 1, treated for 20 minutes daily for 7 days with 2 per cent chloramine-T solution. × 140.

FIG. 5. Section of the skin of the ear of Rabbit 2, Experiment 1 (Fig. 2), treated for 20 minutes daily for 7 days with 0.5 per cent sodium hypochlorite (bleach-HCl-$NaHCO_3$). × 140.

(Cullen and Taylor: Irritant properties of chlorine antiseptics.)

(Cullen and Taylor: Irritant properties of chlorine antiseptics.)




(Cullen and Taylor: Irritant properties of chlorine antiseptics.)

# SPIRILLA ASSOCIATED WITH DISEASE OF THE FETAL MEMBRANES IN CATTLE (INFECTIOUS ABORTION).

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PLATES 81 AND 82.

(Received for publication, September 24, 1918.)

In the course of investigations into the pathology and etiology of abortion in cattle as it prevailed among the animals composing a certain group of herds under one management, and with more or less intercourse between individual herds, there was encountered a fair number of cases from which *Bacillus abortus* Bang could not be isolated from the fetus or placenta either in cultures or through guinea pigs or with both methods combined. These cases ranged themselves into several groups and comprised (*a*) the few fetuses yielding sterile cultures, (*b*) those yielding rapidly growing cultures of colon-like and other bacteria, and (*c*) those yielding pure cultures of a spirillum. It is this latter organism which will be dealt with in this paper.

After much of the work here reported had been done, a report of an English Committee on abortion in sheep came to the writer's notice.[1] The actual work is credited to Sir J. McFadyean and Sir Stewart Stockman. The direction of the investigation was in the hands of a larger committee of which the two authors mentioned were members. The brief report of this committee summarizes the results of the experiments reported in full in the appendix.

The microorganism is described as a vibrio and the more precise nomenclature left for future determination. The vibrio was cultivated on the well known medium consisting of agar, gelatin, and serum in deep layer as first used by Bang[2] for *B. abortus*, and it grew in much the same way as this bacillus. A careful reading of the statements concerning the cultural characters of the

[1] Great Britain Board of Agriculture and Fisheries, Report of the departmental committee on epizootic abortion, London, 1913, 22.

[2] Bang, B., *Z. Thiermed.*, 1897, i, 241.

vibrio does not reveal any essential differences between this organism and the spirillum under discussion. Nor do we find any differences in their negative effects on small laboratory animals. Seven head of cattle were infected with exudate from aborting ewes either with or without the addition of pure cultures to the pathologic material. In two instances the mode of introducing the virus was by mouth, in the rest by intravenous inoculation. Two cases aborted, and in one of these the vibrios were detected. These results are as yet too meager and indefinite to answer the important question whether the vibrio of ovine abortion is the same as the spirillum of the bovine disease.

What is of more interest at the moment is a statement, quite buried in the report, of the isolation of vibrios from cases of abortion in cattle in a herd in Ireland and in Wales in 1911. A brief paragraph is all that is given of these findings and we are left to conjecture whether vibrios were found later in other herds.

### *Lesions Associated with the Spirillum.*

In all, fourteen cases have come under our observation from which pure cultures of the spirillum have been obtained. At the same time the abortion bacillus of Bang was not met with in any of these cases.

Taking the cases consecutively as they occurred in the group of herds under observation since the earliest isolation of the spirillum, beginning June, 1917, and ending September, 1918, there were twenty-seven cases from which the bacillus of Bang was isolated and fourteen cases associated with spirilla. These figures are not to be considered accurate for the reason that a certain small number of cases were not studied and hence are not included. A certain number could not be studied so thoroughly as the rest and hence the associated organism may have been overlooked. Nevertheless, the figures may be taken as indicating that, in this group of animals as elsewhere, *Bacillus abortus* is at present the predominating organism in infections of the fetal membranes of cattle.

The cases examined indicate that the lesions associated with this organism are largely if not exclusively restricted to the fetal membranes and that the fetus suffers secondarily from a gradually increasing interference with the placental circulation. This is precisely what occurs when *Bacillus abortus* invades the chorion and cotyledons. We would therefore expect the changes in the fetus to be much the same in both etiological types. This is the case. It is impossible to

foretell whether a given fetus will yield cultures of *Bacillus abortus*, or of the spirillum, or none at all.

Unfortunately, the fetal membranes are either retained and discharged in a disintegrated and decomposed state, or if discharged with or soon after the fetus they are mostly in more or less advanced stages of autolysis, the death of the fetus having taken place some time previously. The material, therefore, which is the primary seat of the disease will be available in the best state only when cattle are slaughtered and the membranes examined fresh, *in situ*. Such cases are relatively rare and perhaps encountered only by accident. It may be that serological tests will enable us to select specifically affected animals during life.

The main lesions affecting the fetus are edema of the subcutaneous tissue and effusions into the large serous cavities. The fluids are, as a rule, more or less heavily tinged with blood. This effusion is frequently associated with delicate, loose, shreddy deposits of fibrin or more rarely heavy, whitish pseudomembranes in abdomen and much less abundantly on pleura and epicardium. The visceral changes are chiefly those referable to autolytic changes following death. Focal lesions are not present.

The stomachs usually contain considerable quantities of what appears to be swallowed meconium or perhaps, at least in some instances, material driven into the fourth stomach from the small intestines by antiperistaltic movements. The stomachs of normal fetuses contain a colorless, translucent, very thick, viscid fluid which in older fetuses may contain a few pellets of meconium and perhaps some hairs. The diseased fetus, however, almost invariably has in its stomachs a very turbid, thick, yellowish, flaky fluid. Not infrequently there are also found small, whitish, soft, disc-like masses which can be traced back to epithelial excrescences of the amnion and which have come away and been swallowed by the fetus with the amniotic fluid.

The lungs may or may not be inflated, depending on the age of the fetus. In some the air tubes contain fluid identical with that in the stomachs. This fluid may completely fill the trachea and bronchi.

The kidneys are usually surrounded by a thick bloody fluid which collects in a thin layer under the capsule and separates this from the cortex. The tissue about the kidneys is also frequently distended with fluid suffused with blood-coloring matter.

The study of sections of fixed and hardened tissues of fetuses has not brought out anything characteristic of the infection. In the digestive tract the epithelial coverings may be partly or wholly desquamated, probably not as the result of any disease process, but following death of the fetus. Focal lesions were absent here and from the other organs. The lungs in some cases presented collections of cells of undetermined type in the alveoli and smallest air tubes. This absence of active disease processes in the fetus is probably to be accounted for by facts to be submitted later on concerning the habitat of the spirillum.

About the fetal membranes, little that is definitely known can be said until cases are available in which the fetus and membranes can be studied fresh and *in situ*. In general, however, it may be stated that the focal lesions of the cotyledons and of the chorionic membrane between cotyledons, and the exudate, which characterize the cases infected with *Bacillus abortus*, are absent in the cases associated with spirilla. The histological picture resembles in certain respects only that of the disease due to *Bacillus abortus*. The chorionic epithelium is destroyed and the tissue immediately below it is densely infiltrated in spots with cells, whose nuclei are in a state of fragmentation, as if a strong chemotactic influence were at work in the uterochorionic space. There is, furthermore, edema of the chorion and extensive, rather loose cell infiltration into the edematous zone. The nature of these cells, necrotic in the cases examined, has not been definitely made out. In several instances there were dense collections of polynuclear leucocytes among the denuded villi of the cotyledons.

### *Morphological and Biological Characters of the Spirilla.*

The only microorganism thus far encountered in films and sections of the fourteen cases is a spirillum, if we exclude certain mixed infections of the fetus with *Bacillus coli*, miscellaneous bacteria

attaching themselves to the placenta as it is discharged into the bedding and manure, and those swallowed by the fetus over 7 months of age during and just after birth.

Spirilla have been detected microscopically both in the fetus and in the few placentas available, but not in every case from which pure cultures were obtained. If we grant that the seat of the disease is in the fetal membranes, more particularly the chorion, the spirilla make their way into the amniotic fluid and thence into the digestive and respiratory tracts of the fetus. Spirilla have been detected in films of stomach contents in five cases and in sections of the small intestine in two. In the placental tissues they have been seen either alone or with miscellaneous filth bacteria. Their precise habitat in the chorion cannot be fixed upon with the meager material available. In one case the endothelium of certain capillaries of the chorion was crowded with spirilla (Fig. 7). This condition has not been seen in the few other cases examined. In one other case the necrotic cores of the villi contained dense colonies of minute organisms which were probably spirilla. In several other cases spirilla were loosely scattered through the necrotic tissues. Inoculation of guinea pigs in the two latter instances showed that *Bacillus abortus* was absent. It should be stated that the short forms of the spirillum are very minute and when packed together in dense masses only those on the periphery may be recognizable.

In form the spirilla found in fixed and hardened tissues vary more or less in length. The short forms, consisting of 1 and $1\frac{1}{2}$ wave lengths or windings, predominate; those of 2 to 4 windings are less numerous. In the small intestines they occur in the desquamated epithelium and along the free margins of the villi (Fig. 1). There is evidence from the microscopic picture that still shorter forms exist which, owing to their minuteness, are not definable. The transverse diameter or thickness is probably not over 2 $\mu$, the windings about 2 $\mu$ long, amplitude, 0.5 $\mu$.

The importance of a suitable staining agent in disclosing these forms cannot be overestimated. Thus far alkaline methylene blue has given satisfactory results for films. Giemsa's stain, now on hand, has been less so. In fixed material eosin-methylene blue has been used with good results. Bacteria in tissues and fluids may be in

various stages of active multiplication and degeneration and of encapsulation. A given dye may therefore pick out only certain stages. This is probably true of methylene blue. Films of stomach fluid stained 24 to 48 hours in one case showed a dense tangle of long, rather feebly stained spirilla with and without stained granules (Fig. 2), whereas the 10 minute preparation showed only small numbers of fairly short, well stained forms. The spirillum is Gram-negative.

Spirilla from cultures do not present any features not found in the microorganisms in the host tissues and fluids. In the earliest growth the short forms predominate. As the culture grows older longer spirochete-like forms appear and become most numerous. At the same time, deeply stained granules may appear at spaced intervals in some of the spirilla. These are also found in individuals from body fluids and only in the long forms. These facts point towards degenerative processes but further studies may tend to contradict this view.

If bits of tissues or particles of meconium or drops of stomach contents containing spirilla are added to slanted agar and the tube is hermetically closed with sealing wax, there will be seen after 3 or 4 days, along the lateral margins of the slanted surface, between agar and glass, a narrow grayish line extending up from the condensation water one or several centimeters. At the same time or some days later a very thin, barely visible film of growth will have extended from this line inward between agar and glass. In some instances this film continues to spread until it meets the film growing in from the opposite side. Rarely a thin film starts upward on the slope for a few millimeters from the condensation water. If a transfer to a fresh tube of agar is made after 4 or 5 days, a similar though feeble growth may be obtained. A second transfer is, however, apt to fail.

In casting about for media suitable for the continued cultivation of the strains it was found that while tubes containing bits of guinea pig or other mammalian tissue are suitable, the best method was to add a few drops of defibrinated horse blood to the condensation water of ordinary nutrient agar slants. This medium has served the purpose very well and at the time of writing strains from twelve of

the fourteen cases from which spirilla have been isolated in pure culture are still alive. Some have been transferred over 60 times. In general the older strains grow more profusely. After the strains had been under cultivation for some time they were tried on plain agar, sealed, with the result that the oldest strains have grown and are continuing to multiply in the absence of blood or bits of tissue (Figs. 3 and 4).

The growth on blood agar varies somewhat from strain to strain but the main features are the same. In the older strains, multiplication occurs in a thin layer over the sedimented corpuscles in the condensation water, between glass and agar, as a grayish layer on the slope starting from the condensation water and as isolated colonies on the slope. The latter may be absent. When present, they vary in size from mere points to 2 mm. in diameter and have the ordinary appearance of bacterial colonies. On plain agar the growth is much less vigorous as a rule, but even in this medium occasionally the amount of growth is unexpectedly large. Quite invariably the growth in the condensation water resembles a drop of thick translucent mucus and, owing to its viscidity, it may be difficult to pull it away from the inside of the tube in transferring to a fresh tube. Growth also occurs between glass and agar and less frequently on the slope.

The usual cultural media, including carbohydrate media in fermentation tubes, have been tried without success. It is not improbable that after prolonged cultivation in sealed tubes of plain agar it may be possible to obtain cultures in bouillon, milk, etc.

In young cultures the spirilla are in active motion. The short forms, 1 to $1\frac{1}{2}$ windings long, move with great rapidity across the field of the microscope. The longer, more sluggish individuals of two to four windings move in a straight line, revolving about the longitudinal axis at the same time. The very long forms, often bent or curved, are very sluggish and move in irregular manner, about one end, or only revolve. Cultures more than a week old may contain no active individuals.

With this method of cultivation the spirilla have been isolated in pure culture, and quite uniformly so, from the fourth stomach, meconium, and lung tissue of the fetus. They were isolated not so

frequently from spleen, liver, and kidneys. In all cases bits of tissue as large as peas were transferred to the culture tube of plain agar. The invasion of the body generally is thus not the rule and may be due to accidentally favorable conditions.

The action of this organism on the small laboratory animals so far tried is apparently without harmful effects. Of the forty-odd guinea pigs inoculated subcutaneously and intraperitoneally with body fluids and tissues containing cultivable spirilla, all have thriven like normal animals. Several rabbits treated repeatedly with large doses injected into the abdominal cavity have remained well. No anaphylactic symptoms have developed. Rats and mice are also refractory to large doses of cultures. Birds have not yet been tested.

The resistance of the culture forms to spontaneous disintegration is evidently low. Cultures renewed after a period of a week may be lost. On the other hand, some resist. Thus of two original cultures from body tissues and fluids, one was still alive after $2\frac{1}{2}$ months. It was kept at room temperature and protected from drying by sealing wax. Another culture under the same conditions and only $3\frac{1}{2}$ weeks old was dead.

Owing to the time required to bring the various strains of spirilla to grow actively in media free from blood and tissues, serological tests to determine whether the strains react alike to immune sera are still under way and they will be described in a later publication. Preliminary tests with the serum of rabbits repeatedly inoculated with large doses of living spirilla indicate a close relationship of the fetal strains.

### *Probable Relation of the Spirillum to the Disease Process.*

The proof of any direct etiological relation between a given microorganism and a definite disease process is brought by reproducing the latter with pure cultures of the microorganism in question. This should not be a difficult undertaking were it not that infectious abortion is widespread. To utilize for crucial tests animals from infected herds may lead to errors of interpretation in two directions. The inoculated animals may abort on account of spontaneous infection or they may have acquired more or less resistance and fail to

abort after inoculation. The experimental tests with the spirillum are therefore waiting for suitable subjects. In the meantime, the presumption that this microorganism bears a relation to abortion similar to that borne by *Bacillus abortus* is supported by several facts.

The spirillum is isolated in pure culture from fetuses under precisely the same conditions as is *Bacillus abortus*. The cultural peculiarities of the two in requiring a reduced oxygen tension are the same. The distribution of both organisms topographically is the same. Both occur quite regularly in the gastrointestinal and respiratory tracts of the fetus, more rarely in other viscera. Both occur in the placental fluids but *Bacillus abortus* is more readily demonstrable because of the susceptible guinea pig, which eliminates in itself the miscellaneous filth bacteria in the discharged fetal membranes.

The cases in which the spirillum was demonstrated did not harbor *Bacillus abortus*. None of the guinea pigs inoculated with fetal material or placenta has acquired the specific lesions produced by it. On the other hand, the spirillum was absent in the cases from which *Bacillus abortus* has been isolated. In the many hundreds of cultures prepared in this laboratory the bacillus of Bang develops after 5 to 10 days. This period gives the spirillum ample opportunity to multiply if present. No such mixed infection has been observed. It appears, therefore, that these organisms are mutually exclusive for reasons not known at present. A further significant fact which cannot be discussed until the cases associated with *Bacillus abortus* have been analyzed and presented is the limitation of the spirillum to second and later pregnancies thus far.

Another fact which supports the etiological rôle of the spirillum is the discovery of vibrios or spirilla in the abortion of sheep and in two instances in herds of cattle in Ireland and Wales, as has been stated above. Although the method of cultivation was different in our work from that used by the English workers, the evidence is sufficiently strong to warrant the inference that they are closely related.

TABLE I.

| Cow No. | No. of sire. | Length of fetus. | Date of breeding. | Date of abortion. | No. of pregnancy after purchase. | Cultures. | | | | | | | Guinea pig inoculations. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 4th stomach. | Meconium. | Lung. | Spleen. | Liver. | Kidney. | Fetal fluids. | 4th stomach. | Lung. | Meconium. | Placenta. |
| | | *in.* | | *1918* | | | | | | | | | | | | |
| 67 | ? | 6½ | (Purchased Sept. 2, 1917.) | Jan. 6 | ? | | | | | | | + | | | | −− |
| | | | | *1917* | | | | | | | | | | | | |
| 90 | ? | 22½ | (Purchased Nov. 29, 1916.) | June 2 | ? | ++ | −−* | | | + | | | | | | |
| | | | *1916* | | | | | | | | | | | | | |
| 95 | 1 | 28½ | Nov. 13 | " 14 | 2nd | | | ++ | | | | | | | | |
| | | | *1917* | | | | | | | | | | | | | |
| 149 | 1 | 16½ | June 10 | Oct. 27 | 2nd | + | | ++ | | | | | − | −− | | |
| 159 | 2 | 16¾ | " 16 | Nov 7 | 3rd | Heavy growth. | | +− | − | | Several species. | | − | | | −− |
| 179 | 3 | 29 | Apr. 23 | " 26 | 2nd | +− | | | − | | − | | − | − | | |
| | | | | *1918* | | | | | | | | | | | | |
| 192 | | 36 | May 30 | Jan. 23 | 3rd | ? | ++ | +++ | −− | −− | −− | | | − | − | |
| 213 | 4,5 | 21 | Aug. 4 | Mar. 18 | 4th | ++ | | ++ | −− | −− | −− | | − | − | − | |
| | | | " 31 | | | | | | | | | | | | | |
| | | | *1918* | | | | | | | | | | | | | |
| 246 | 2,6 | 7½ | Jan. 11 | May 28 | 2nd | | | ++− | | ++− | | | | − | −† | |
| | | | " 24 | | | | | | | | | | | | | |
| 251 | 2 | 17½ | " 12 | June 10 | 2nd | ++ | ++* | −+− | | | | | − | − | | |
| 256 | 6 | 19 | " 14 | " 18 | 4th | ++ | | + | ++ | ++ | ++ | | − | − | | |
| | | | *1917* | | | | | | | | | | | | | |
| 258 | 7 | 27 | Dec. 16 | " 29 | 2nd | ? | ++ | + | ++ | ++ | ++ | | | −− | −− | |
| | | | *1918* | | | | | | | | | | | | | |
| 263 | 3 | 20 | Feb. 9 | July 30 | 3rd | ++ | ++ | +++ | | ++ | | | − | − | − | |
| 267 | 4 | 23 | " 14 | Aug. 12 | 3rd | ++ | ++ | ++ | ++ | ++ | ++ | | − | − | − | |

* Small intestine.

† Liver.

SUMMARY.

Spirilla of identical morphological and cultural characters have been isolated in pure culture from the fetuses of fourteen cases of abortion. The condition of the fetus is much the same whether spirilla or the bacilli of abortion are present. This condition is probably due in both cases to interference with the placental circulation. The injurious action of the etiological factor when spirilla are present is limited to the fetal membranes, more particularly the chorion. Definite lesions of the fetus were not detected. The spirilla gain access to the digestive and respiratory organs of the fetus when the latter swallows the amniotic fluid. More rarely they are disseminated through the body, probably through the circulation. The spirilla will grow in certain culture media only under reduced oxygen tension, readily secured by sealing the ordinary culture tubes with sealing wax. Laboratory animals (mammals) are refractory. The precise relation of the spirillum to the pathologic process remains to be more definitely formulated. Since the spirillum was first isolated, twenty-seven cases have been found associated with *Bacillus abortus* and fourteen with the spirillum. In none was a mixed infection with both organisms detected. The spirillum has been isolated only from the second or succeeding pregnancies.

Table I summarizes the data collected thus far. It gives by number the male and shows that the spirillum is not associated with any one bull. The spirillum has been found in fetuses of various ages as shown in Column 3. The distribution of spirilla as shown by cultures is given, the sign + indicating pure cultures, the sign − no growth. The guinea pig inoculations are shown to be uniformly negative as regards *Bacillus abortus*.

APPENDIX.

Inasmuch as no data have thus far been published concerning the occurrence of spirilla in bovine abortion it has seemed best to publish the following brief notes on the individual cases.

*Cow 90.*—Cow purchased November 29, 1916. Aborted June 2, 1917. Placenta discharged soon after. Both examined June 4 after having been refrigerated.

Male fetus, length 57 cm. Hairless, except on lips. Marked general subcutaneous edema with slight staining of fluid with hemoglobin. In abdomen and pleural and pericardial sacs considerable amounts of blood-tinged fluid together with thin elastic fibrinous deposits on liver, omentum, lungs, and heart. All organs are more or less soft, putty-like in consistency.

Stomachs contain a turbid, bile-stained, flaky viscid fluid. Cultures made by adding a few drops of stomach contents and a bit of liver to agar and sealing. In these, active spirilla found in pure culture.

Two guinea pigs inoculated with 1.3 cc. of a suspension in salt solution of cotyledons, ground in sand. Chloroformed after 2½ months. No lesions of *B. abortus*. Cultures from spleen remain sterile.

Sections of the placenta fixed in Zenker's fluid and stained in eosin-methylene blue show a complete loss of the epithelium covering chorion and villi of cotyledons and the presence of groups of polynuclears at the roots of villi. In certain blood vessels of chorion from 10 to 120$\mu$ in diameter the endothelial cells are crowded with minute bacteria, not resembling *B. abortus* and in a situation where the latter is not found (Fig. 7). The minute organisms are probably short spirilla, for on the thin margin of groups, where they lie in single layer, short curved forms are recognizable. Endothelial cells within the lumina of these vessels are also crowded with the same forms. Where chorion and amnion are fused there is considerable edema with infiltration of cells no longer identifiable (necrotic).

*Twin Fetuses 94 and 95.*—Dropped on pasture June 14, 1917. Placenta was discharged some hours later but unfortunately was not brought to the laboratory with the fetuses.

*Fetus 94.*—Male fetus with good coat of hair, black and white; length 72 cm. No hemorrhagic discoloration or edema of subcutis. Mouth soiled with earth. Stomachs contain a thick, very viscid, dark greenish, opaque, flaky fluid. Liver large, rather pale; kidneys soft, with numerous cortical hemorrhages. Lungs indicate that some air has entered. Nothing noteworthy otherwise. Cultures from this fetus as follows: Those from spleen, kidney, and lungs remain free from growth. From this case no spirilla isolated. Sections from fixed tissues show nothing noteworthy beyond congestion.

*Fetus 95.*—Twin of No. 94. Abdominal organs, except spleen and liver, eaten out by some animal on pasture. Lungs contain a trifle air, but are otherwise heavy and full of blood.

Of five tubes prepared with bits of lung tissue, three developed into pure cultures of a spirillum. The two remaining contained spore-bearing aerobes.

Histological examination of the lung tissue shows presence in air tubes and alveoli of groups of cells of uncertain character. Interlobular tissue broadened and lymph spaces filled with blood.

*Cow 149.*—Cow aborted October 27, 1917. Due to calve March 19, 1918. Calved normally in 1916. Placenta now retained.

Fetus 16½ inches long, male. Skin readily pulled from underlying tissues exposing axillary and inguinal regions as if dissected by *B. welchii*. All muscles soft and readily torn away from attachments. Blood-tinted fluid in abdominal cavity. Liver macerated and tissue readily crushed. In the stomachs a pinkish fluid. The layer of epithelium detached and carried out by incision with the fluid like sheets of tissue paper.

Blood-tinted fluids in pleural sacs; lungs with slight interlobular edema. No odors of bacterial decomposition.

Pure cultures of a spirillum obtained from bits of lung tissue and stomach fluid. Cultures from other organs not made.

Two guinea pigs inoculated with suspensions of lung tissue and one with stomach contents. They were chloroformed after 7 weeks. No lesions found and all cultures from spleens remain sterile.

*Cow 158.*—This case is reported because it also was probably associated with spirilla, but cultures were not made. *B. abortus* was absent.

Cow purchased February 16, 1917. Calved normally March 10 and aborted November 6.

Fetus and membranes discharged together, the two still connected by umbilical cord. No odor of decomposition. Fetus 12½ inches long, female. In subcutis of ventral aspect and especially over pubis a translucent, only in part blood-tinted edema. Blood-tinted fluid in abdomen. Liver very friable. Thorax contains considerable blood-tinted fluid.

The placenta shows a glass-clear gelatinous edema between chorion and amnion. No liquid obtainable by incision into this layer. Cotyledons whitish; the villi long and slender when floated in water.

Three guinea pigs inoculated with 1 cc. of a turbid suspension of cotyledons ground up in salt solution were kept 8 weeks. They were then chloroformed and cultures prepared from bits of spleen tissue. All remained free from growth. The guinea pigs were normal.

Sections of fixed and hardened cotyledons show extensive collections of polynuclears between the villi denuded of epithelium. The villi are represented only by the necrotic connective tissue stroma. In this stroma there are exceedingly dense masses of minute bacteria which in places thin out and are resolved into short wavy forms.

*Cow 159.*—This cow was purchased in April, 1914. Calved normally in 1915. Aborted February 23, 1916 and November 7, 1917.

The fetus of 1916 was 3 to 4 inches long and discharged with membranes. It had undergone considerable maceration, the entire body being soft, semitranslucent and only head, limbs, and ribs recognizable. Odor peculiar but not suggestive of bacterial decomposition. Two guinea pigs inoculated with placental tissues remained well and were chloroformed after 10 weeks. No lesions were found and spleen cultures remained indefinitely free from growth. Sections of the placenta show groups of polynuclears among villi, but no spirilla among other bacteria.

The fetus and placenta of 1917 were obtained together. Fetus 16¾ inches long, male. Blood-tinted fluid in subcutis, in abdomen, perirenal tissue, thorax, and pericardial sac. A thin deposit of fibrin shreds on heart surface. Rumen contains a thick, viscid, pinkish fluid and a mass resembling the color and consistency of yolk of egg. Fourth stomach contains a similar fluid but no suspended matter.

The placenta received only in part. After removing shavings and other bedding, the cotyledons were found to be gray to whitish. There was no edema of the fetal membranes. The amniotic fluid still remaining was thin, slightly blood-tinted, with fine flakes in suspension.

Cultures were made from contents of fourth stomach and amniotic fluid. Only one from stomach contents showed growth in the form of a pure culture of spirilla.

Sections of fixed and hardened tissues of fetus presented nothing noteworthy. Spirilla were detected in contents of small intestine. Sections of the chorion, including portions of cotyledons, showed infiltration of cells into the wall of the small blood vessels. The cells are probably mononuclear but the bizarre form of the nuclei makes it impossible to define them. On the periphery of the cell infiltration are many curved forms, of one to two windings, very minute. They are not in dense groups but scattered singly through the tissue. None is seen in the endothelium of the vessels as is the case in No. 90. There is some fibrin in the vascular walls and also in the nearly nude villi of the cotyledons. Polynuclears are absent but this may be due to the washing of the fetal membranes necessitated by the filth attached to them. Miscellaneous bacteria are, as might be expected, attached to the free exposed surfaces of the fetal membranes.

Three guinea pigs were inoculated, one with suspension of ground up cotyledons, one with contents of fourth stomach, and one with amniotic fluid. After 8 weeks they were chloroformed. Cultures from the spleens remained free from growth.

*Cow 179.*—Cow aborted November 26, 1917. The fetus was placed in refrigerator and examined November 28. Placenta retained.

Fetus 74 cm. long, with a good coat of hair. Surface of body stained yellowish. Some shavings from bedding in mouth. Abdomen somewhat distended.

Stomachs filled with a yellowish, only slightly viscid fluid, holding in suspension abundant soft yellowish brown masses. Large intestine well distended with meconium. Liver slightly enlarged, yellowish. Spleen on section shows some lighter areas. Lungs not air-distended. Trachea and bronchi filled with stomach contents.

Cultures were made directly from fetal tissues. Three tubes inoculated respectively with contents of fourth stomach, spleen, and kidney tissue remained sterile. In one kidney and lung tube heavy growths appeared of several species. In one tube of stomach contents a pure culture of spirilla developed. In one liver tube a small capsulated bacillus appeared. Sections of various organs, fixed and hardened, show nothing characteristic or noteworthy.

Two guinea pigs inoculated, one with a suspension of ground up lung tissue, the other with stomach contents, were chloroformed after 7 weeks. Lesions absent. Cultures from spleen negative.

*Cow 67.*—Fetus, within membranes. Discharged January 6, 1918, and placed at once into refrigerator. Examined January 7.

Fetus about 17 cm. long. Head resting on abdomen and directed caudad. Cervical vertebræ separated and only skin and sternomastoid muscle holding head to body. Tissues soft and partly autolyzed. Allantoic and amniotic fluids turbid, reddish in color.

A culture from amniotic fluid added to slanted agar developed into a pure culture of a spirillum.

No guinea pigs inoculated from this case.

*Cow 192.*—Cow aborted January 23, 1918. Due March 18. Calved 1914 and 1917. Fetus received, frozen, at laboratory. Placenta retained. Length 91 cm., weight 45 pounds. The only noteworthy features of this case are as follows:

The stomachs contained much very viscid light brownish fluid, suspending pieces of meconium up to 7 cm. long, and some hairs. Colon and rectum distended with very dark, tenacious meconium.

Lungs inflated with exception of the left ventral lobe.

Films from surface of mucosa of fourth stomach and rectum negative as regards bacteria.

Cultures from the various organs gave the following result. Two liver, two spleen, and two kidney tubes remained free from growth. Three tubes containing lung tissue and two containing meconium developed into pure cultures of a spirillum. Both tubes containing stomach contents developed heavy growths with gas formation. (Evidently the fetus had breathed and swallowed at birth.)

Two guinea pigs inoculated, one with lung tissue, the other with meconium, were kept 5 weeks, then chloroformed. Lesions and spleen cultures negative.

*Cow 213.*—Cow calved in 1914, 1915, and 1916. Aborted March 18, 1918. Due June 9. Placenta discharged soon after.

Male fetus, 53 cm. long, hairless. Pelvis unusually narrow; nature of deformity not investigated. Muscular and subcutaneous tissue slightly edematous. Abdomen contains some blood-stained fluid.

Stomachs not overdistended. Rumen contains a thick, tomato sauce-like fluid, viscid, holding in suspension whitish flakes 2 to 3 mm. in diameter, consisting of squamous epithelium (small cell masses from inner surface of amnion). Films show a few spirilla of three to four windings. Fourth stomach contains a still homogeneous, very viscid, slightly amber-colored, transparent fluid, which had to be cut with scissors on account of viscidity, in order to get some for cultures. Rectum distended with small cylindrical masses of dry meconium packed side by side.

Liver of a coarsely mottled yellowish appearance. Spleen several times normal size, flabby. Only one kidney present, this with unusually broad cortex.

Thorax half full of blood-stained fluid. Lungs not inflated. Slight interlobular edema.

Placenta covered with foreign matter and emitting a strong fecal odor. Cotyledons in the main of normal appearance. Some are grayish to whitish. The outstanding feature of the chorion is the presence of slightly elevated patches of an irregular nodular surface usually 3 to 4 cm. in diameter. The nodules are very firm, not readily crushed or pulled away from underlying tissue. Subchorionic tissue edematous. Films from different cotyledons and patches show several varieties of bacteria including spirilla of 1½ to 4 windings. These are more numerous than the other miscellaneous bacteria. Of the cultures prepared two kidney, two spleen, and one liver tube remain sterile. Pure cultures of spirilla are obtained from two lung and two fourth stomach tubes.

Three guinea pigs inoculated respectively with suspensions of lung tissue, contents of fourth stomach, and meconium were chloroformed after 6 weeks and found normal. Of the spleen cultures two tubes contain spore formers, the rest remain sterile.

Microscopic examination of fixed tissues of fetus presents nothing noteworthy. Sections from various regions of placenta show marked edema of chorion and a zone of cell infiltration and cell necrosis between chorion and amnion. The epithelium of chorion has disappeared and the bared margin is densely infiltrated with cells in localized areas. The cells are evidently necrosed, for the nuclei are pyknotic and of bizarre shapes. Some smaller vessels of chorion are nearly closed by lamellated thrombi. Bacteria not detected in these sections. Fibrils of fibrin abundant in tissue spaces and vessels and mask or simulate the presence of bacteria. On the denuded surface of chorion some thick bacilli, and seen only once, a dense mass of spirilla attached to a projecting shred of tissue. The villi of cotyledons not appreciably altered, but most of epithelium has disappeared.

*Cow 246.*—This cow was purchased September 1917 and aborted in November. This fetus was not obtained. She aborted again during the night of May 27, 1918. Fetus obtained early next morning and refrigerated until May 30.

Fetus still within unbroken membranes. About 20 cm. long. A thick, chocolate-colored fluid within amnion. Skin of fetus easily drawn away exposing partially macerated muscular tissue. Organs in same condition, the liver resembling thick cottage cheese in consistency.

Tubes were inoculated only from liver and lungs. In these pure cultures of spirilla developed.

Two guinea pigs inoculated with a suspension of lungs and liver mixed were chloroformed after 6 weeks. Lesions and spleen cultures negative.

*Cow 251.*—This cow aborted June 10, 1918. Due October 21. The fetus was found in the manure drop in the morning. Placenta retained.

Fetus female, hairless, 44 cm. long, weight 2,510 gm. Some coils of small intestine hanging out of opening about umbilicus from which blood-tinted fluid escapes. Universal blood-tinted edema of subcutis, of the musculature, and of perirenal space. Liver large, tissue discolored, partly macerated. Stomachs

contain a small quantity of a very turbid yellowish fluid containing whitish particles, composed of squamous cell masses. Large intestines contracted, empty. Thorax half full of blood-stained fluid. Air tubes free from aspirated matter.

Of the tubes inoculated pure cultures of spirilla developed from contents of fourth stomach, small intestine, and lungs. Lung tubes also contained heavy growths in condensation water.

Two guinea pigs were inoculated with suspension of lung tissue and contents of fourth stomach respectively. Chloroformed after 6 weeks, they were found normal and spleen cultures remained sterile.

*Cow 256*.—Cow purchased in 1914. Calved normally in 1915, 1916, and 1917. Aborted June 17, 1918. Due October 23. Placenta retained.

Fetus, male, length 47 cm., weight 3,925 gm. Hairless. General slight subcutaneous edema, blood-tinted. Large bleb on back containing 95 cc. of blood-stained fluid. 240 cc. of bloody fluid in abdomen and about 50 cc. in pleural sacs. Stomachs contain a thick turbid yellowish fluid. Spirilla detected in films (Fig. 5). Nothing noteworthy about remaining viscera.

Tubes inoculated with bits of tissue and fluids yielded the following results. Two tubes with stomach contents developed mixed cultures of spirilla and other bacteria. Two spleen tubes developed into pure cultures of spirilla. The same is true of two kidney, one lung, and two liver tubes. One other lung tube had a very heavy growth.

Two guinea pigs inoculated, one with stomach contents, the other with lung tissue, were kept 5½ weeks, then chloroformed. They were normal and the spleen cultures remained free from growth.

Sections of fixed and hardened tissues show the presence of numerous short spirilla in four different regions of small intestines. They were limited to the contents.

*Cow* 258.—Cow purchased August, 1917. Calved in same year. Aborted July 1, 1918. Due September 24. Placenta retained.

Fetus hairless, male, length 57 cm., weight 22 pounds. Abdomen prominent. Testicles swollen, edematous. Subcutaneous edema very slight. In abdomen about 500 cc. of a turbid light reddish fluid, depositing on staining a white sediment made up of leucocytes. Covering liver and adjacent organs is a heavy elastic whitish pseudomembrane, spongy, 1 to 2 mm. thick on liver, and easily stripped off. It contains many cells, appearing as mononuclears in films. Stomachs contain a very viscid fluid, suspending yellowish brown flakes and masses of squamous cells. Liver rather large, yellowish, friable.

Small amount of blood-tinted fluid in pleural sacs. Scattering light yellowish foci in lungs, 2 to 3 mm. in diameter, air-containing. The rest airless. Pericardial cavity contains a turbid, nearly colorless fluid. Epicardium covered with a thin, whitish roughened exudate, giving the heart a furry appearance. Films show spirilla in contents of fourth stomach and rectum.

Tubes of agar inoculated with bits of organs and with fluids from stomachs, etc., gave the following results. Two tubes from fourth stomach and one from

lungs heavily overgrown. Two liver, one lung, two spleen, two kidney, and two meconium tubes develop into pure cultures of spirilla.

Four guinea pigs inoculated, two with meconium and two with lung tissue, were kept 6 weeks and then chloroformed. All were normal and spleen cultures remained free from growth.

In sections of the various organs no noteworthy changes. Spirilla not detected.

*Cow 263.*—Cow purchased in September 1915. Calved in 1916 and 1917. Aborted July 30, 1918. Due November 18.

Fetus 51 cm. long, hairless except on lips and chin. Cord remains about 12 cm. long, swollen, edematous. A piece of meconium 5 cm. long protrudes from rectum.

Abdomen contains blood-tinted fluid. Liver slightly softened. Under capsule of kidneys a thin layer of a thick bloody fluid.

Mouth contains a little yellowish, flaky material. Stomachs normally distended with a viscid fluid suspending soft yellowish granules and whitish flakes representing cellular excrescenses of amnion. Films from contents show some short and long spirilla.

Thorax partly filled with a bloody fluid. Trachea contains fluid like that in stomachs.

Cultures from different organs give the following results. Two spleen and two kidney tubes remain sterile. Two stomach, two liver, three lung, and two meconium tubes develop into pure cultures of spirilla.

Three guinea pigs inoculated respectively with contents of fourth stomach, meconium, and lung tissue kept 6 weeks were free from lesions when chloroformed. Cultures from spleens negative.

*Cow 267.*—Cow purchased July, 1916. Calved 1916 and 1917. Aborted August 12, 1918. Due November 23. Placenta retained.

Fetus, male, hairless, 59 cm. long, weight 6,618 gm. Marked enlargement of abdomen. General subcutaneous edema causing swollen appearance of limbs. Testicles swollen, edematous.

Abdomen contains 650 to 700 cc. of a turbid reddish fluid. White, elastic pseudomembranes cover liver almost entirely and extend over omentum and mesenteries. Easily pulled away.

Stomachs contain a thick, turbid, not very viscid, yellowish fluid. Films show presence of spirilla. Large intestine well filled with meconium. Spirilla present.

Kidneys contain about a dozen cysts, 2 or 3 mm. in diameter, at base of medulla. Spleen with pulp rather soft.

Pleural sacs contain about 50 to 75 cc. of a deeply blood-stained fluid. Minute shreds of whitish exudate sprinkled over all surfaces of the lungs. The air tubes contain a yellowish mucoid fluid like that in stomachs. Spirilla present (Fig. 6).

Cultures of various tissues show the following results. Two lung, two kidney, two spleen, two liver, two fourth stomach, and two meconium tubes develop into pure cultures of spirilla.

Three guinea pigs inoculated respectively with contents of fourth stomach, lung tissue, and meconium and kept over 5 weeks remained well. When they were chloroformed no lesions were found and spleen cultures remained sterile.

## EXPLANATION OF PLATES.

All films are stained in alkaline methylene blue.

### PLATE 81.

FIG. 1. Section of small intestine, Fetus 256. The figure includes only the contents made up of desquamated cells and three spirilla in focus. Many others, not in view, are present in the cell mass. Eosin-methylene blue. × 1,000.

FIG. 2. Film from contents of trachea of Fetus 267, containing large numbers of long spirilla, brought out by prolonged staining (48 hours). The material consists of regurgitated and aspirated stomach contents. × 1,000.

FIG. 3. Fresh agar culture of spirilla from Fetus 179. Eleventh transfer. × 1,000.

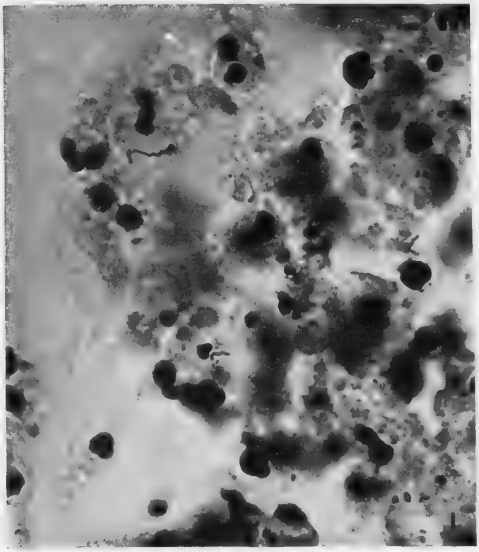
FIG. 4. Old agar culture of spirilla from Fetus 149. Fourteenth transfer. The spirilla are long and beset with deeply stained coccus-like bodies. × 1,000.

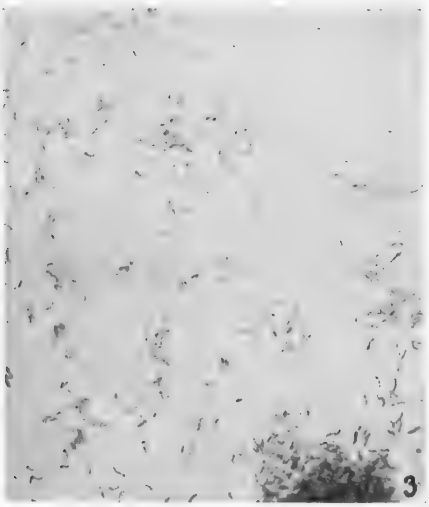
### PLATE 82.

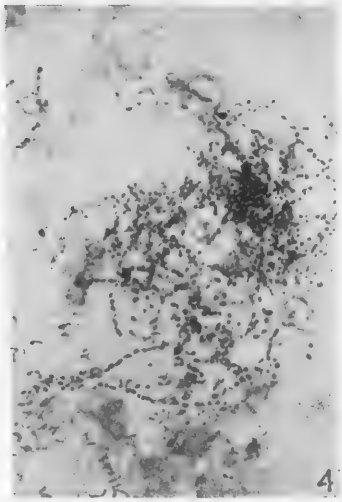
FIG. 5. Stained film of contents of rumen, Fetus 256, four spirilla and one degenerated cell in view. × 1,000.

FIG. 6. Two spirilla in film from air tubes of Fetus 267. × 1,000.

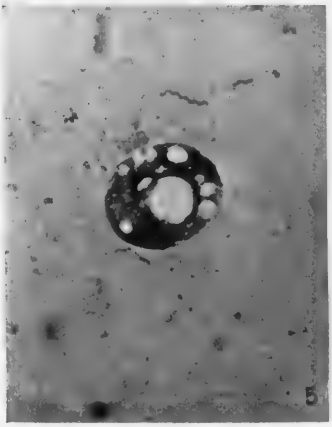
FIG. 7. Transverse section of chorion of Fetus 90, showing the endothelial cells of a blood vessel packed with bacteria which in thin spots have been identified as short spirilla. Cells within lumen of vessel are densely packed with them. Eosin-methylene blue. × 1,000.

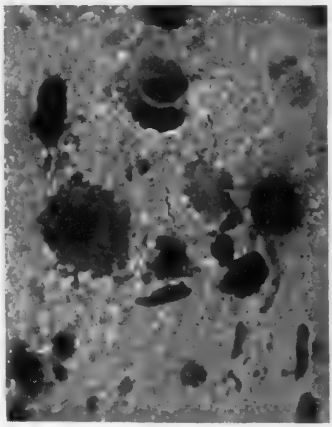


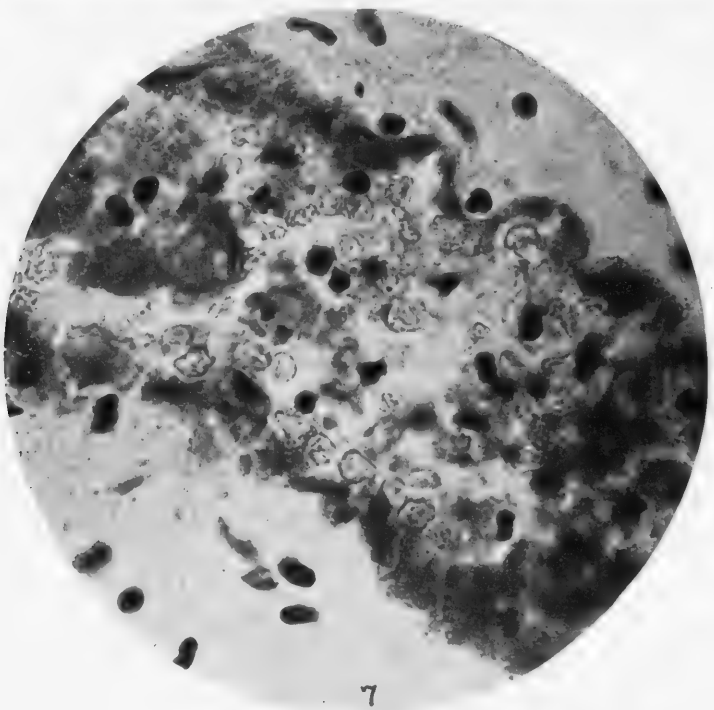




(Smith: Spirilla in infectious abortion of cattle.)






(Smith: Spirilla in infectious abortion of cattle.)

# STUDIES IN BOVINE MASTITIS.

## III. Infection of the Udder with Micrococci and Other Microorganisms.

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### INTRODUCTION.

Many of the earlier investigators noted the presence of micrococci in certain types of udder inflammation. Lucet[1] cultivated gelatin-liquefying, Gram-staining micrococci from seven cases. Guillebeau[2] in studying 85 cases of mastitis found that in 52, micrococci were responsible for more or less severe inflammations. He divided them into two broad groups—the staphylococci which liquefied gelatin and the galactococci which failed to do so. The staphylococci were more frequently found than the galactococci. Steiger[3] reported micrococci associated with udder inflammation in sixteen cases. Like Guillebeau, he classified them as staphylococci and galactococci. Both classes of cocci were described as having a diameter of 1 micron and staining by Gram's method. They also coagulated milk. The staphylococci produced a grayish white, yellow, or golden brown pigment on potato. The liquefaction of gelatin was constant. The galactococci failed to liquefy gelatin but usually produced pigment on potato. Steiger's observations on the pathogenicity of micrococci for cows are interesting. He described eleven cases in cows and one in a goat which cleared up spontaneously within periods varying from 3 days to 3 weeks. On the other hand, his protocols show that streptococci produce a much more severe type of disease. Of the ten cows infected with streptococci, eight suffered from severe mastitis which became chronic.

Savage[4] appears to have been the first to describe in detail the biological characters of the micrococci associated with mammitis. Of the 31 instances which he reported five were associated with staphylococci. He states that the proof

[1] Lucet, *Rec. méd. vét.*, 1889, vi, series 7, 423.

[2] Guillebeau, *Landswirtsch. Jahrb. Schweiz.*, 1890, iv.

[3] Steiger, P., *Centr. Bakteriol., 1te Abt., Orig.*, 1904, xxxv, 326, 467, 574.

[4] Savage, W. G., *Rep. Med. Off. Local Gov. Bd. 1907–08*, xxxvii, 425.

of the causal relation of staphylococci to mastitis is unsatisfactory, especially since staphylococci are frequently found in the normal udder. Savage, too, noted the apparent mildness of the disease they produced.

### *Micrococci Isolated from the Udder.*

Of 81 instances of mastitis studied, twenty-four were associated with micrococci. As a rule, the processes which micrococci excite are mild, but in a few instances there has been a serious, acute involvement which became chronic.

The following record is typical of one of the more severe types of infection.

*Cow 60.*—Dec. 10, 1916. Gave birth to a calf. Sept. 17, 1917. Mastitis developed in the left hind quarter.

Sept. 18. The left hind quarter was swollen, exceedingly firm, and tender. Milk could only be withdrawn with difficulty. Milking was accomplished with considerable pain at first but the discomfort subsided as the pressure was relieved. The milk was yellowish white and thickened with many large flocculi. On standing, many of the larger particles coalesced, forming a large viscid mass on the bottom of the bottle and an excess of fat surmounting a watery liquid. Blood agar plate cultures prepared from the milk revealed 2,172,000 colonies per cc. The surface colonies at the end of 24 hours were 1.5 mm. in diameter, round, and raised. At the end of 48 hours they were larger and had developed a typical orange pigment. The deep colonies were small, round, or ovoid and nonhemolytic.

Sept. 19. The quarter was still swollen, firm, and tender. The milk was yellow and flocculent.

| | |
|---|---|
| Cells per cc. | 16,000,000 |
| Cocci " " | 220,000 |

The other quarters appeared normal and the milk from them was free from flocculi. Cell counts were well within normal limits. Plate cultures failed to reveal chromogenic micrococci.

Sept. 23.

| | |
|---|---|
| Cells per cc. | 26,000,000 |
| Cocci " " | 560,000 |

From Sept. 23 to 28 the inflammation subsided rapidly. The quarter became more flaccid; a few irregular firm nodules were observed about the milk cystern. The milk was normal in color but still contained a few fine flakes.

| | |
|---|---|
| Cells per cc. | 10,400,000 |
| Cocci " " | 1,100 |

Dec. 2. The quarter appeared normal. The milk was free from flocculi.

| | |
|---|---|
| Cells per cc | 760,000 |
| Cocci " " | 1,500 |

Frequently one observes mild cases associated with only a slight inflammation of the udder. The infected quarter may be slightly enlarged, a trifle firm, and secrete flocculent milk for a day or two. The micrococci usually reach a maximum number when the inflammation is at its height. The number decreases rapidly after the crisis is reached and frequently they disappear from the milk.

Micrococcic infections are not always mild. In a number of instances they have been observed in cases of considerable severity.

Cow 80 developed a severe inflammation of the right hind quarter on September 20, 1917. The quarter was at first enlarged, hot, and painful. The acute inflammation subsided but the quadrant atrophied. The secretion was much reduced and consisted of purulent milk. 6 months later the general condition of the right hind quarter remained unchanged.

Cow 81 calved September 30, 1916. Mastitis developed in the right hind quarter on November 20. After the acute inflammation had subsided a more chronic one continued. The quarter decreased in size, became very firm, and secreted only a few cubic centimeters of flocculent milk. The micrococci were found in the secretion from the affected quarter 5 months later.

In addition, chromogenic micrococci have been isolated from three cases of abscess of the udder. The abscesses were situated in the subcutis and tended to break down and discharge externally. Reparative processes soon set in and the condition cleared up without treatment. Usually they occurred with greatest frequency in cows that came into the herd a short time before. In all probability the infection is spread from animal to animal during the processes of grooming and washing before milking.

*Cultural Characters of Micrococci Isolated from Inflamed Udders.*

It seemed advisable to cultivate the micrococci in the same media employed in the studies of streptococci. Tubes containing 13 cc. of 1 per cent solutions of the carbohydrates and other substances were used to test the fermentative action of the strains (Table I).

TABLE I.

*Morphological and Biological Characters of the Micrococci Isolated from Inflamed Udders.*

| No. of culture. | Grouping. | Gram's stain. | Chromogenesis on agar. | Milk. | Production of acid in. | | | | | | | | Liquefaction of gelatin. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Dextrose. | Lactose. | Saccharose. | Maltose. | Raffinose. | Inulin. | Mannite. | Salicin. | |
| | | | | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | |
| C.60 | Clumps. | + | Orange. | Firmly coagulated. | 3.1 | 3.0 | 3.1 | 2.9 | 0.9 | 0.8 | 3.2 | 0.8 | + |
| M.W. | " | + | Yellowish white. | " " | 3.2 | 2.9 | 3.3 | 2.5 | 1.6 | 1.5 | 2.6 | 1.1 | + |
| M.4 | " | + | Orange. | " " | 3.0 | 2.9 | 2.5 | 2.4 | 0.8 | 1.0 | 2.8 | 1.0 | + |
| M.9 | " | + | " | " " | 3.0 | 3.0 | 2.6 | 2.3 | 0.7 | 1.0 | 2.2 | 0.8 | + |
| M.27 | " | + | " | " " | 3.2 | 2.8 | 2.9 | 2.8 | 0.7 | 0.7 | 2.7 | 0.6 | + |
| M.32 | " | + | Grayish white; translucent. | Coagulated on boiling. | 3.5 | 3.2 | 2.9 | 2.8 | 1.1 | 0.8 | 2.1 | 0.7 | + |
| M.39 | " | + | Orange. | Firmly coagulated. | 4.1 | 3.4 | 3.6 | 3.2 | 0.7 | 0.6 | 3.4 | 0.6 | + |
| A.5 | " | + | Yellowish white. | " " | 3.2 | 2.8 | 2.7 | 2.8 | 0.9 | 0.9 | 3.0 | 1.1 | + |
| A.8 | " | + | Orange. | " " | 3.2 | 2.4 | 2.4 | 2.7 | 0.8 | 0.9 | 2.9 | 0.9 | + |
| A.19 | " | + | " | " " | 4.0 | 2.8 | 3.0 | 3.8 | 0.6 | 0.6 | 2.6 | 3.6 | + |
| M.Z. | " | + | White. | Coagulated on boiling. | 2.7 | 2.9 | 2.9 | 2.7 | 0.9 | 0.9 | 1.8 | 0.7 | + |
| M.M. | Clumps. | + | Yellowish white. | Partially coagulated. | 3.5 | 3.1 | 3.2 | 1.9 | 0.0 | 0.0 | 2.5 | 0.0 | + |
| M.1 | " | + | Orange. | Firmly " | 3.6 | 3.0 | 3.0 | 3.0 | 0.0 | 0.0 | 1.9 | 0.0 | + |
| M.15 | " | + | White. | " " | 3.5 | 3.2 | 3.4 | 2.2 | 0.0 | 0.0 | 1.5 | 0.0 | + |
| M.20 | " | + | " | " " | 3.9 | 3.8 | 3.8 | 3.5 | 0.0 | 0.0 | 1.6 | 0.0 | + |
| M.68 | " | + | " | " " | 3.7 | 2.9 | 3.1 | 2.5 | 0.0 | 0.0 | 2.5 | 0.0 | + |
| M.70 | " | + | Yellowish white. | " " | 4.0 | 3.2 | 3.3 | 2.1 | 0.0 | 0.1 | 3.1 | 0.0 | + |
| M.72 | " | + | White. | " " | 3.5 | 3.6 | 3.5 | 2.1 | 0.0 | 0.0 | 3.6 | 0.0 | + |
| M.83 | " | + | Orange. | " " | 3.1 | 2.7 | 2.6 | 2.5 | 0.1 | 0.0 | 1.7 | 0.0 | + |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M.N. | Clumps. | + | White. | Firmly coagulated. | 3.4 | 3.3 | 3.2 | 2.1 | 0.2 | 0.1 | 0.2 | 0.1 | + |
| M.2 | " | + | " | Partially " | 3.8 | 3.5 | 4.0 | 3.5 | 0.0 | 0.1 | 0.0 | 0.1 | + |
| M.19 | Clumps. | + | Yellowish white. | Firmly coagulated. | 3.6 | 3.2 | 3.2 | 2.9 | 0.1 | 0.0 | 2.5 | 1.0 | + |
| M.49 | Clumps. | + | White. | Firmly coagulated. | 3.1 | 2.9 | 2.7 | 1.8 | 1.0 | 0.1 | 1.9 | 0.1 | + |
| M.O. | Pairs. | + | White. | Firmly coagulated. | 5.7 | 3.4 | 4.0 | 4.1 | 2.3 | 2.1 | 3.2 | 2.1 | − |
| M.V. | " | + | Grayish white. | Acid. | 2.6 | 2.1 | 2.4 | 2.4 | 0.6 | 0.2 | 1.8 | 2.3 | − |
| C.80 | Clumps. | + | " " translucent. | Firmly coagulated. | 3.8 | 3.0 | 3.2 | 3.3 | 0.0 | 3.7 | 1.8 | 3.0 | − |
| C.81 | " | + | " " " | Coagulated on boiling. | 3.2 | 2.8 | 3.3 | 3.4 | 0.9 | 0.0 | 1.4 | 2.9 | − |
| C.69 | " | + | White. | Firmly coagulated. | 3.0 | 2.8 | 2.6 | 1.8 | 0.1 | 0.0 | 1.3 | 0.0 | − |

The figures given beneath the test substances refer to the actual acid production after 5 days incubation at 38°C. Gelatin tubes were incubated at 22°C. for 14 days. Liquefaction is indicated by the sign +.

It will be seen that the micrococci fall into the same broad groups that were noted by Lucet, Guillebeau, Steiger, and others. They termed those liquefying gelatin as staphylococci and those failing to do so as galactococci. From the table it will be readily observed that the larger group is composed of staphylococci. Savage also described both types of micrococci. Further comparison between the cultural characters of the micrococci isolated by the earlier workers and those indicated in the table is impossible. Earlier descriptions were based upon the character of the growth on potato and in gelatin, milk, and bouillon. Savage, however, studied the action of twenty-two strains, isolated from five cases of mastitis, in broth containing maltose, lactose, glycerol, and mannite, as well as in milk. All his strains fermented maltose and glycerol. All but one produced acidity in lactose. Sixteen fermented mannite. In nineteen instances gelatin was liquefied.

In addition to their action upon gelatin many strains possess other minor differential characteristics. Of the twenty-three strains that liquefy gelatin, eleven produce acid in all the test substances. Eight ferment dextrose, lactose, saccharose, maltose, and mannite. Two other species fail to ferment raffinose, inulin, mannite, and salicin. Cultures M.19 and M.49 differ from all the others. The former produces acid in dextrose, lactose, saccharose, maltose, mannite, and salicin. The latter differs only in its failure to ferment salicin.

The number of non-gelatin-liquefying micrococci is too small to warrant any final grouping.

Evans[5] found micrococci in 58.8 per cent of samples of milk drawn directly from the udder. In describing the fermentative action of the staphylococci, dextrose, lactose, maltose, raffinose, mannite, and glycerol were used as test substances, and for this reason comparisons are difficult. There is a great similarity existing between staphylococci isolated from normal and inflamed udders.

A few strains of staphylococci have been tested for their pathogenicity. 1 cc. of 24 hour bouillon cultures of Culture M.M. injected

[5] Evans, A. C., *J. Infect. Dis.*, 1916, xviii, 437.

into the ear vein of a rabbit produced a fatal septicemia within 24 hours. Cultures C.60 and M.15 produced severe involvement of the kidneys; Culture M.72 caused a slight rise in temperature for a few days following the injection. Strains M.N., M.O., and C.80 failed to produce ill effects.

*Mastitis Associated with Bacillus coli and Bacillus lactis aerogenes.*

Many have described udder inflammations caused by *B. coli communis*. Guillebeau believed at first that he was dealing with a new species. Later, however, Jensen[6] identified Guillebeau's bacilli as *B. coli*. Guillebeau, Jensen, Steiger, and others pointed out that the colon group is responsible for extremely severe mastitis. Savage observed only milder types of infection produced by this organism. Zwick and Weichel,[7] Gilruth and Macdonald,[8] and others have reported outbreaks of mastitis caused by *B. lactis aerogenes*.

Udder inflammations due to this class of organisms have been relatively infrequent in the material at my disposal. The udders of two animals became invaded with *Bacillus coli*. In another instance *Bacillus lactis aerogenes* produced a severe inflammation of one quarter.

*Description of Cases Attributed to Colon Bacilli.*

*Cow 58.*—Calved Mar. 19, 1917. July 18. Mastitis developed in the left hind quarter. The quarter was firm, although not appreciably enlarged. The milk was normal in color but contained many small, white flocculi in suspension.

Cells per cc..... 7,500,000
Organisms per cc..... 790 (pure culture of *B. coli*).

The milk from the other quarters appeared normal.

July 20. The left hind quarter was more flaccid. The milk appeared a trifle watery and contained a few flocculi.

Cells per cc..... 1,800,000
Organisms per cc..... 100

*B. coli* was not observed in the plates.

The other quarters appeared normal. Plate cultures failed to develop *B. coli*.

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[6] Jensen, C. O., *Ergebn. allg. Path. u. path. Anat. 1897*, 1899, iv, 830.
[7] Zwick and Weichel, *Arb. k. Gsndhtsamte.*, 1910, xxxiv, 391.
[8] Gilruth, J. A., and Macdonald, N., *Vet. J.*, 1911, lxvii, 217.

July 22. The milk was again examined. All the samples were normal and failed to contain *B. coli*.

Another cow became infected with *Bacillus coli* in the right hind quarter. The quarter was swollen, firm, hot, and painful. The secretion resembled hemolyzed blood and contained large plugs of viscid grayish red material which coalesced on standing into a solid mass on the bottom of the bottle. The secretion contained 264,000 bacilli per cc. The condition became chronic and the animal was slaughtered.

### *Infection of the Udder with Bacillus lactis aerogenes.*

*Cow 68.*—Calved July 22, 1917. Mastitis was first observed in the left hind quarter on Nov. 11. The quarter appeared normal except for an irregular firm nodule 5 cm. in diameter situated about the milk cystern. The milk was watery and contained large numbers of grayish white flocculi. Plate cultures revealed 26,000 organisms per cc. The surface colonies were round, raised, grayish white, and slimy in appearance. They measured from 3.5 to 4 mm. in diameter after 48 hours incubation. The deeper colonies were round or ovoid.

This cow was under observation for several months. For the first 2 weeks the nodule about the cystern increased in size until it involved the lower third of the quarter. Later it began to decrease, and finally the quarter became flaccid. The secretion gradually declined until only a few cubic centimeters could be withdrawn. Cell counts were not satisfactory, since the flocculi coalesced after washing and centrifugalization. On July 23 the highest cell count of 8,000,000 was recorded. The plate cultures on that day revealed 20,000 colonies of *B. lactis aerogenes* per cc. of milk. The organisms showed a tendency to diminish. On Dec. 17 only 580 colonies were found in a cubic centimeter of milk. The infection persisted throughout the lactation period. The other quarters were not affected.

The cultural characters of *Bacillus coli* and *Bacillus lactis aerogenes* are given in Table II.

### *Infection of the Udder with Gram-Staining Rod-Shaped Organisms.*

In four cases of more or less severity Gram-positive rods have been the only organisms present. In three instances the only apparent symptoms were a transient thickening of the walls of the cystern, usually producing an irregular nodule 4 or 5 cm. in diameter which soon disappeared. The milk was normal except for a varying number of tiny, viscid flocculi. The condition cleared up readily within a few days.

TABLE II.

*Cultural Characters of B. coli and B. lactis aerogenes Isolated from Inflamed Udders.*

| No. of culture. | Motility. | Indol. | Milk. | Dextrose. | Lactose. | Saccharose. | Maltose. | Raffinose. | Inulin. | Mannite. | Salicin. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| C.58 | + | + | Firmly coagulated. | Gas 44% H 3 $CO_2$ 1 Acid 3.9% | Gas 50% H 4 $CO_2$ 1 Acid 3.5% | Gas: a bubble. Acid 1.5% | Acid 3.8% | Acid 2.4% | Acid 0.0% | Acid 2.9% | Acid 1.9% |
| M.P. | + | + | Firmly coagulated. | Gas 50% H 7 $CO_2$ 2 Acid 3.9% | Gas 50% H 3 $CO_2$ 1 Acid 3.0% | Acid 0.0% | Acid 3.6% | Acid 0.0% | Acid 0.0% | Acid 2.9% | Acid 0.0% |
| C.68 | − | − | Firmly coagulated. | Acid 4.2% | Acid 4.1% | Acid 4.2% | Acid 3.6% | Acid 2.5% | Acid 0.1% | Acid 2.8% | Acid 3.2% |

*Cow 55.*—This cow represents a more or less typical example of mild infection. In the preceding period of lactation she had suffered from a severe mammitis of the left hind quarter due to non-hemolytic streptococci. Toward the end of the milking period the condition improved but the streptococci were always present in the milk. The cow gave birth to a calf on Nov. 12, 1917. Plate cultures prepared from the milk failed to contain streptococci. On Dec. 26 she suffered from a mild attack of mastitis in the left hind quarter. The quarter appeared normal except for an irregular firm swelling about the cystern. The milk was watery and contained a few flocculi. Plate cultures prepared from the milk revealed 12,300 tiny colonies per cc. The colonies grew slowly and after 72 hours incubation measured 1 mm. in diameter. The attack lasted a few days. Plate cultures prepared on Dec. 29 showed only 200 colonies per cc. The swelling about the cystern had almost disappeared.

A much more severe infection was met with in Cow 79.

*Cow 79.*—Calved Oct. 8, 1917.

Dec. 27. The animal slipped and fell while walking on the ice. It was supposed that the udder had been injured by the fall, since mastitis made its appearance in the right hind quarter on Dec. 29.

Jan. 8, 1918. The quarter was greatly enlarged, very firm, and hot. Milking caused considerable pain at first but brought relief after a few streams had been withdrawn. The secretion was a thickened greenish white fluid. The odor was offensive. Only 1 pint of milk could be expressed from the quarter. Horse blood agar plate cultures containing 0.00005 cc. of milk revealed countless organisms. Even after the milk had been diluted to 1:50,000 large numbers appeared on the plates after incubation. It was estimated that 700,000,000 colonies developed from 1 cc. of milk. Attempts to count the cells failed because of the unbreakable masses of sediment after centrifugalization. Stained films of milk sediment revealed many polymorphonuclear leucocytes and round cells, and, in addition, large numbers of small rod-shaped organisms and coccoid forms.

Several colonies were fished from the plates and inoculated into serum bouillon. In addition, tubes of melted agar containing a column of medium 8 or 9 cm. deep were inoculated with diluted milk. From the tiny colonies which developed, serum bouillon tubes were inoculated. Plate cultures were prepared from each series of bouillon cultures. In every instance the only organisms developing in the plates were tiny, translucent surface colonies accompanied by deeper biconvex colonies surrounded by a narrow zone of hemolysis. Morphologically all the organisms studied from both series resembled each other.

The condition became chronic. The swelling gradually subsided and ultimately the quarter became smaller than the others. The milk retained its offensive odor.

Mar. 23. The cow was slaughtered. The udder was sent to the laboratory. The right hind quarter was rounded and very firm. On section the lower portion

was principally composed of firm, glistening connective tissue. Many of the larger lactiferous ducts had been obliterated. The milk cystern was constricted. The mucosa of the cystern was necrotic and stained brownish green in an area 2.5 cm. in diameter. Many of the lobules of the upper portion of the gland had been replaced with connective tissue. Others had atrophied. The epithelium was dry and granular.

Microscopically the lesions differed considerably from those found in streptococcic mastitis. Stained sections prepared from the upper portions of the gland revealed, besides an increase of the interlobular tissue, degenerative changes of the epithelium and invasion of leucocytes into the lumen of the acini. Leucocytes were present in considerable numbers in the interacinar vessels. In tissue from the middle and lower portions of the quarter the processes were much more severe. Frequently many lobules had been replaced with connective tissue enmeshing polymorphonuclear leucocytes and round cells. Local areas of hemorrhage had occurred. The connective tissue was edematous in places. A large proportion of the lactiferous ducts had been obliterated. In several the productive process could still be observed. The lining cells of such a duct were compressed. Connective tissue filaments interlacing leucocytes and round cells had invaded the lumen of the vessel. Many newly formed blood vessels were observed in the stroma.

The epithelium of the milk cystern and larger milk ducts had proliferated. The cells had lost their original polyhedral appearance and had become elongated and flattened and resembled squamous epithelium. Finger-like processes frequently extended into the lumen of the duct or into the surrounding connective tissue. Several isolated nests of squamous epithelium were observed in the connective tissue.

## *Morphological and Cultural Characters of Four Strains of Rod-Shaped Organisms Isolated from Inflamed Udders.*

*Culture M.T.*—Morphology: Tiny non-motile rods. Many show beaded or granular staining with Giemsa's stain. Coccoid forms are not uncommon. The rods stain well by Gram's method.

Colonies in horse blood agar plate cultures:

Surface colonies: Small, round, raised, grayish. 0.5 mm. in diameter after 48 hours incubation.

Deep colonies: Spherical or ovoid. Fail to produce hemolysis in horse blood agar.

*Culture C.55 X.*—The same morphological and other characters that are described for Culture M.T.

*Culture M.44.*—Morphology: Very small rods, apparently non-motile. Coccoid forms were not infrequent. Stained preparations revealed many beaded forms. The organisms stain well by Gram's method.

Colonies on horse blood agar:

Surface colonies: Hemispherical, translucent colonies, rarely attaining 0.5 mm. in diameter after 48 hours incubation.

Deep colonies: Ovoid and biconvex surrounded by a narrow hemolytic zone.

*Culture C.79.*—This culture resembles Culture M.44 in its morphological characters. The growth in horse blood agar plate cultures is the same.

Additional cultural characters are given in Table III. It was necessary to add a small amount of sterile horse serum to each tube of media before good growth could be obtained.

TABLE III.

*Biological Characters of Gram-Staining Rod-Shaped Organisms Associated with Mastitis.*

| No. of culture. | Growth in bouillon. | Milk. | Production of acid in. | | | | | | | | Liquefaction of coagulated serum. | Liquefaction of gelatin. | Hemolysis. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Dextrose. | Lactose. | Saccharose. | Maltose. | Raffinose. | Inulin. | Mannite. | Salicin. | | | |
| | | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | | | |
| C.79 | Clear. | Firmly coagulated and partially digested. | 1.6 | 1.1 | 2.9 | 2.1 | 0.6 | 0.5 | 0.5 | 0.3 | + | − | + |
| M.44 | Turbid. | " " | 2.1 | 1.4 | 1.5 | 2.5 | 0.3 | 0.6 | 0.2 | 0.0 | + | − | + |
| M.T. | Clear. | Unchanged. | Fails to change the reaction of the media. | | | | | | | | − | − | − |
| C.55X. | Turbid. | " | " | | | " | | | " | | − | − | − |

None of the cultures proved pathogenic when injected into the peritoneal cavity of white mice or guinea pigs.

## DISCUSSION AND SUMMARY.

Aside from the streptococci, micrococci have been the next most frequent group of organisms isolated from inflamed udders. They produce various types of disease. Some give rise to only a mild catarrh of the larger milk ducts and cystern, while others produce more or less severe parenchymatous inflammation. On the whole, the prognosis is more favorable with micrococcic infection than with

that associated with streptococci. Cases of considerable severity have, however, been attributed to staphylococci.

Micrococci similar in many respects to those associated with mastitis have been found to occur in the normal udder. This has led Savage to question their true etiological significance. In many instances micrococci may gain access to the udder and produce slight disturbances that are entirely overlooked. Even more severe changes may follow infection. After recovery the organisms still remain in the milk. This was observed in the case of Cow 60 infected with staphylococci. One frequently observes the elimination of streptococci from the udder even after apparent recovery from an attack of streptococcic mastitis. Doubtless streptococci and micrococci observed in these udders would be classed as belonging to the normal flora. Even though micrococci do occur in supposedly normal udders Evans has shown that many are pathogenic for rabbits. The introduction of these organisms into the udders of non-resistant individuals might well give rise to more or less intense inflammation. The multiplication would doubtless be rapid until resistance had been established.

In addition to the micrococci two other groups of rod-shaped organisms have been found associated with udder inflammation. In two instances *Bacillus coli* has been isolated from cases of mastitis and in another *Bacillus lactis aerogenes*. In four, tiny motile Gram-staining microorganisms have been obtained in pure culture. Two of these strains (Nos. C.79 and M.44) have been identified as *Bacillus pyogenes*.

# STUDIES IN BOVINE MASTITIS.

## IV. The Sources of Infection in Streptococcic Mastitis.

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### INTRODUCTION.

Much has been written concerning the etiology of mastitis. Lucet, Guillebeau, Steiger, Savage, Henderson,[1] and others have all isolated streptococci from inflamed udders. During the past year, of the 85 cases of mastitis which have been examined, 54 have been associated with streptococci. Thirty-five cows were infected with the non-hemolytic type and seventeen with the hemolytic. In two instances both types were present.

Mastitis is endemic in the herd that has supplied most of the material for this investigation. The disease is causing more serious losses every year. During 1916, 64 animals were disposed of because of diseased udders. In 1917 the losses increased to 71. Aside from the actual number of animals slaughtered there have been a considerable number of milder cases which recovered. The milk from these animals is discarded as long as it contains flocculi. In addition, one or more quarters frequently atrophy after severe infection.

Streptococcic infections have occurred throughout the herd with extreme irregularity. Attempts to trace infection from one animal to another have usually resulted in failure. It seemed that there must be some source of infection aside from the clinical cases. Many have pointed out the danger of carrying infection on the hands of the milker. Even assuming that the milker might transmit the virus from a clinical case to other cows it could only account for a small

[1] Henderson, J., *J. Comp. Path. and Therap.*, 1904, xvii, 24.

number of infections, especially as the milkers are always instructed to exercise caution in milking known cases.

Three possible sources of infection aside from clinical cases were pointed out in previous studies. Briefly, they may be enumerated as follows: (1) Streptococci are eliminated in the milk before symptoms appear. (2) Frequently they remain in the udder after the disease has subsided. (3) Streptococci may gain access to the udder and persist for a time and then disappear without causing clinical manifestations of mastitis.

Williams[2] and others have suggested the genital tract as a source of infection and point out that mastitis often follows retention of the placenta, metritis, etc. Hagin[3] in bacteriological examinations of the placentas of twenty cows was able to cultivate streptococci from the uterochorionic space in six instances. It is assumed that during parturition these organisms are washed down with the fluids over the buttocks, udder, and teats. They may thus gain access to the teat canal.

With these points in view the milk and the vaginal secretion of 50 cows in one barn in which mastitis had occurred with considerable frequency during the past year were examined. It was postulated that streptococci isolated either from the udder or from the vagina must possess cultural characters and agglutination affinities in common with those previously found associated with mastitis before they could be considered of etiological significance. The writer[4, 5] in previous papers has shown that the streptococci found in udder inflammations produced a uniform acidity in dextrose, lactose, saccharose, and maltose. Many fermented salicin, others failed to do so. Milk was always coagulated. In addition, all the non-hemolytic streptococci were agglutinated by an antiserum produced by the immunization of an animal with one strain. The hemolytic types were likewise agglutinated with their specific serum. To conform to the mastitis type, then, it was assumed that a streptococcus must produce acid in dextrose, lactose, saccharose, and maltose. Fer-

[2] Williams, W. L., *Cornell Vet.*, 1918, viii, 63.

[3] Hagin, W. A., *Ann. Rep. New York State Vet. College, Cornell Univ.*, 1916–17, 140.

[4] Jones, F. S., *J. Exp. Med.*, 1918, xxviii, 149.

[5] Jones, F. S., *J. Exp. Med.*, 1918, xxviii, 253.

mentation of salicin was considered variable. It should coagulate milk and be agglutinated by its group serum (hemolytic or non-hemolytic).

*Examination of Milk Drawn Directly from the Udders.*

Milk was always drawn directly into a sterile wide mouthed bottle. The cows were brushed, washed, and dried before the sample was drawn. The first few streams were discarded. A sample from each quarter was milked into the same bottle. The milk was iced at once and usually plated within an hour. 1 cc. of milk was added to 9 cc. of sterile 0.9 per cent sodium chloride solution and shaken vigorously. 1 cc. of this mixture was added to 12 cc. of 2 per cent veal infusion agar and poured into a Petri dish containing 1 cc. of defibrinated horse blood and the whole mixed. After 24 hours incubation subcultures were made from colonies resembling streptococci. The various media were inoculated from the subcultures which had been proved to be streptococci. Table I shows the arrangement of the cows in the barn and those harboring streptococci in the udder.

From Table I it will be noted that sixteen cows were eliminating streptococci in the milk. During the observation which extended from March until June, 1918, mastitis developed clinically in Cows 10, 21, and 23. Records of the cows showed that during the lactation periods Cows 7, 8, 10, 23, and 37 suffered from mastitis. They had apparently recovered before the milk examinations were begun. The milk from Cows 6, 13, 20, 22, 25, 26, 41, and 50 had never been rejected because of udder inflammation. It must be recognized that such conditions may have been so mild that they escaped detection.

In Tables II and III the morphological and biological characters of the non-hemolytic and hemolytic streptococci isolated from the udders of these apparently normal cows are given. The figures given in the column beneath the various test substances represent the net amount of acid produced in a 1 per cent solution after 5 days incubation.

Since both the non-hemolytic and hemolytic streptococci isolated from the udder were identical in their cultural characters with those

TABLE I.

*Arrangement of the Cows in the Barn and Those Harboring Streptococci in the Udder.*

| Colonies per cc. of milk. | Per cent of streptococci. | No. of subculture. | No. of cow. | No. of cow. | Colonies per cc. of milk. | Per cent of streptococci. | No. of subculture. |
|---|---|---|---|---|---|---|---|
| 1,600 | 40 per cent hemolytic. | E.25 | 50 | 1 | 900 | None. | |
| 1,370 | None. | | 49 | 2 | 1,280 | " | |
| 600 | " | | 48 | 3 | 1,640 | " | |
| 1,250 | " | | 47 | 4 | 2,800 | " | |
| 60 | " | | 46 | 5 | 3,100 | " | |
| 230 | " | | 45 | 6 | 8,000 | 10 per cent non-hemolytic. | E.2 |
| 60 | " | | 44 | 7 | 4,000 | 50 per cent hemolytic. | E.3 E.4 |
| 90 | " | | 43 | 8 | 9,600 | Pure culture hemolytic. | E.5 |
| 20 | " | | 42 | 9 | 4,700 | None. | |
| 8,580 | 75 per cent non-hemolytic. | E.23 | 41 | 10 | 5,760 | Pure culture hemolytic. | E.26 E.6A E.7 |
| 480 | None. | | 40 | 11 | 500 | Pure culture non-hemolytic. | E.8 |
| 30 | " | | 39 | 12 | 220 | None. | |
| 280 | " | | 38 | 13 | 400 | Pure culture non-hemolytic. | E.9 |
| 9,800 | 70 per cent non-hemolytic. | E.19 | 37 | 14 | 20 | None. | |
| 1,700 | None. | | 36 | 15 | 900 | " | |
| 140 | " | | 35 | 16 | 850 | " | |
| 50 | " | | 34 | 17 | 780 | " | |
| 1,060 | 95 per cent hemolytic. | E.17 | 33 | 18 | 70 | " | |
| 480 | None. | | 32 | 19 | 6,100 | " | |
| 1,300 | " | | 31 | 20 | 930 | Pure culture hemolytic. | E.10 |
| 600 | " | | 30 | 21 | 166,000 | Pure culture non-hemolytic. | E.11 |
| 50 | " | | 29 | 22 | 3,750 | 95 per cent hemolytic. | E.12 |
| 6,850 | " | | 28 | 23 | 20,800 | 25 " " non-hemolytic. | E.13 |
| 30 | " | | 27 | 24 | 2,200 | None. | |
| 1,100 | 40 per cent hemolytic. | E.15 | 26 | 25 | 16,000 | 80 per cent hemolytic. | E.24 |

associated with mastitis, it became necessary to test their agglutinative affinity with their specific group serum. The results of these tests are recorded in Tables IV and V.

TABLE II.

*Morphological and Biological Characters of the Non-Hemolytic Streptococci Isolated from the Udders of Cows in Table I.*

| No. of culture. | Grouping in bouillon. | Gram's stain. | Growth in bouillon. | Milk. | Production of acid in. | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Dextrose. | Lactose. | Saccharose. | Maltose. | Raffinose. | Inulin. | Mannite. | Salicin. |
| | | | | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| E.2 | M.C.* | + | Turbid. | Firmly coagulated. | 4.1 | 3.6 | 3.3 | 3.8 | 0.0 | 0.0 | 0.0 | 3.4 |
| E.8 | " | + | " | " " | 4.1 | 3.3 | 3.2 | 3.1 | 0.0 | 0.1 | 0.0 | 2.4 |
| E.9 | L.C. | + | Clear. | " " | 4.5 | 3.8 | 3.9 | 4.2 | 0.0 | 0.0 | 0.1 | 3.9 |
| E.11 | M.C. | + | Turbid. | " " | 4.6 | 3.6 | 3.7 | 3.8 | 0.0 | 0.0 | 0.0 | 3.9 |
| E.13 | L.C. | + | " | " " | 4.6 | 3.9 | 3.8 | 4.4 | 0.0 | 0.1 | 0.2 | 2.9 |
| E.19 | " | + | " | " " | 4.6 | 3.6 | 3.5 | 4.0 | 0.0 | 0.0 | 0.0 | 3.5 |
| E.23 | M.C. | + | " | " " | 4.5 | 3.9 | 3.6 | 3.9 | 0.1 | 0.1 | 0.0 | 3.7 |
| E.24 | L.C. | + | " | " " | 4.5 | 4.0 | 3.9 | 3.7 | 0.0 | 0.1 | 0.0 | 1.6 |

*The length of chains has been indicated as follows: L. C., chains of more than 20 elements; M. C., chains composed of 8 to 20 cocci; S. C., chains of less than 8.

TABLE III.

*Morphological and Biological Characters of the Hemolytic Streptococci Isolated from the Udders of Cows in Table I.*

| No. of culture. | Grouping in bouillon. | Gram's stain. | Growth in bouillon. | Milk. | Production of acid in. | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Dextrose. | Lactose. | Saccharose. | Maltose. | Raffinose. | Inulin. | Mannite. | Salicin. |
| | | | | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| E.3 | L.C. | + | Turbid. | Firmly coagulated. | 4.2 | 3.8 | 3.9 | 4.2 | 0.0 | 0.1 | 0.1 | 3.0 |
| E.5 | " | + | Clear. | " " | 4.5 | 4.2 | 3.8 | 3.7 | 0.0 | 0.0 | 0.0 | 3.0 |
| E.6 | " | + | " | " " | 4.7 | 3.9 | 3.7 | 3.7 | 0.0 | 0.0 | 0.0 | 3.2 |
| E.6A. | " | + | " | " " | 4.7 | 4.1 | 3.8 | 3.6 | 0.0 | 0.0 | 0.0 | 2.4 |
| E.7 | " | + | " | " " | 4.4 | 3.7 | 3.8 | 3.7 | 0.0 | 0.0 | 0.0 | 2.4 |
| E.10 | M.C. | + | Turbid. | " " | 4.6 | 4.0 | 3.8 | 3.9 | 0.0 | 0.0 | 0.0 | 3.1 |
| E.12 | " | + | " | " " | 4.9 | 4.1 | 4.0 | 4.4 | 0.0 | 0.1 | 0.0 | 0.0 |
| E.15 | L.C. | + | Clear. | " " | 4.0 | 3.5 | 3.3 | 3.3 | 0.0 | 0.0 | 0.0 | 3.0 |
| E.17 | " | + | " | " " | 3.7 | 4.0 | 3.8 | 3.8 | 0.1 | 0.0 | 0.0 | 2.9 |
| E.25 | " | + | " | " " | 4.4 | 3.6 | 4.0 | 4.0 | 0.1 | 0.0 | 0.1 | 3.7 |

TABLE IV.

*Agglutination Titer of the Non-Hemolytic Streptococci Isolated from the Udder When Tested with the Serum of a Cow Immunized with a Single Strain of Non-Hemolytic Mastitis Streptococci.*

| No. of culture. | Dilution of serum. | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1:100 | 1:500 | 1:1,000 | 1:2,000 | 1:5,000 | 1:10,000 | 1:20,000 |
| Mastitis streptococcus used in immunization. | +++* | +++ | +++ | +++ | +++ | +++ | ++ |
| E.2 | +++ | ++ | + | + | ? | − | − |
| E.8 | +++ | +++ | +++ | +++ | +++ | ++ | + |
| E.9 | +++ | +++ | ++ | + | − | − | − |
| E.11 | ++ | + | + | − | − | − | − |
| E.13 | +++ | +++ | +++ | +++ | +++ | +++ | ++ |
| E.19 | +++ | +++ | +++ | +++ | +++ | + | ? |
| E.23 | +++ | +++ | +++ | +++ | +++ | ++ | + |
| E.24 | +++ | +++ | ++ | + | − | − | − |

* +++ indicates complete agglutination, ++ considerable clumping without entire clearing of the fluid, + a moderate agglutination, − no clumping. A control tube of bacterial suspension to which serum was not added was prepared and incubated in each series.

TABLE V.

*Agglutination Titer of the Hemolytic Streptococci Isolated from the Udder When Tested with the Serum of a Rabbit Immunized with a Single Strain of Hemolytic Mastitis Streptococci.*

| No. of culture. | Dilution of serum. | | | | |
|---|---|---|---|---|---|
| | 1:100 | 1:200 | 1:500 | 1:1,000 | 1:2,000 |
| Mastitis streptococcus used in immunization. | +++ | +++ | +++ | +++ | ++ |
| E.3 | +++ | +++ | + | − | − |
| E.4 | +++ | +++ | +++ | +++ | + |
| E.5 | +++ | +++ | +++ | ++ | + |
| E.6 | +++ | +++ | ++ | + | − |
| E.6A. | +++ | +++ | +++ | + | − |
| E.7 | +++ | +++ | +++ | ++ | + |
| E.10 | +++ | +++ | +++ | ++ | + |
| E.12 | +++ | +++ | +++ | ++ | + |
| E.15 | +++ | +++ | +++ | + | − |
| E.17 | +++ | +++ | ++ | + | − |
| E.25 | +++ | +++ | + | − | − |

In addition to the udder, the genital tract of cows was considered a possible source of infection. Mohler and Traum[6] while investigating contagious abortion made bacteriological examinations of the vaginæ of nine normal cows. A considerable number of species of organisms were identified, among them *Streptococcus pyogenes bovis* and *Streptococcus pyogenes albus*. Detailed descriptions of the characters of each species were not given.

Bacteriological examinations of the vaginæ of the 50 cows previously referred to and others that had recently aborted or passed through normal parturition were made. Sterile swabs were inserted into the vagina for a distance of 6 or 8 cm. Within an hour these swabs were agitated for a few seconds in a tube containing 9 cc. of 0.9 per cent sterile sodium chloride solution. After shaking the suspension briskly for a few seconds three platinum loopfuls of the mixture were inoculated into 12 cc. of melted agar cooled to 45°C. The melted agar was poured into a Petri dish containing 1 cc. of sterile defibrinated horse blood and after mixing thoroughly, incubated for 24 hours at 38°C. Subcultures were made from colonies resembling streptococci.

In all, 34 strains of non-hemolytic streptococci have been isolated from the vaginæ of 64 cows. In no instance have hemolytic streptococci been found.

The morphological and cultural characters of all the streptococci isolated from the vagina of cows are given in Table VI. They have been arranged in groups according to their action upon the various test substances.

As a matter of convenience, grouping on the basis of salicin fermentation has not been carried out, especially as acid production by mastitis streptococci in salicin has not been constant. The largest group, representing seventeen strains, attacks mannite. These streptococci have not been found to be associated with mastitis in any of the material studied. The next largest group of eleven strains fails to attack raffinose, inulin, and mannite, and approaches more nearly the types of streptococci which I have isolated from

[6] Mohler, J. R., and Traum, J., *U. S. Dept. Agric., Bureau Animal Industry, 28th Ann. Rep.*, 1911, 147.

TABLE VI.

*Morphological and Biological Characters of the Non-Hemolytic Streptococci Isolated from the Vagina of 64 Cows.*

| No. of culture. | Grouping. | Gram's stain. | Growth in bouillon. | Milk. | Production of acid in. | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Dextrose. | Lactose. | Saccharose. | Maltose. | Raffinose. | Inulin. | Mannite. | Salicin. |
| | | | | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| V.9 | M.C. | + | Clear. | Coagulated on boiling. | 2.7 | 2.5 | 2.7 | 2.6 | 0.0 | 0.0 | 1.7 | 0.0 |
| V.15 | " | + | " | " " " | 2.1 | 1.7 | 2.3 | 1.8 | 0.1 | 0.0 | 1.9 | 1.9 |
| V.20 | Pairs and S.C. | + | Turbid. | Partially coagulated on boiling. | 2.1 | 2.3 | 2.3 | 2.3 | 0.0 | 0.0 | 1.9 | 2.2 |
| V.26 | S.C. | + | Clear. | Coagulated on boiling. | 3.4 | 2.8 | 3.2 | 2.9 | 0.0 | 0.0 | 2.7 | 2.3 |
| V.27 | M.C. | + | Turbid. | " " " | 3.7 | 3.5 | 3.5 | 3.1 | 0.0 | 0.0 | 2.0 | 3.1 |
| V.28 | " | + | " | " " " | 3.4 | 3.0 | 3.3 | 3.1 | 0.0 | 0.0 | 2.3 | 2.6 |
| V.30 | S.C. | + | Clear. | Acid 1.2 per cent. | 2.2 | 2.1 | 2.1 | 1.7 | 0.0 | 0.0 | 1.9 | 0.0 |
| V.33 | L.C. | + | Turbid. | Coagulated on boiling. | 3.9 | 3.6 | 3.8 | 3.4 | 0.0 | 0.0 | 2.3 | 3.3 |
| V.34 | M.C. | + | Clear. | " " " | 3.2 | 2.8 | 3.0 | 3.0 | 0.0 | 0.0 | 2.0 | 2.8 |
| V.35 | L.C. | + | Turbid. | " " " | 2.6 | 2.5 | 2.9 | 3.0 | 0.0 | 0.0 | 0.7 | 1.9 |
| V.36 | M.C. | + | " | " " " | 2.5 | 2.7 | 2.7 | 2.4 | 0.0 | 0.0 | 1.6 | 0.0 |
| V.37 | " | + | " | " " " | 2.8 | 2.6 | 2.7 | 2.6 | 0.0 | 0.0 | 1.6 | 0.0 |
| V.38 | S.C. | + | " | " " " | 2.7 | 2.6 | 2.7 | 2.5 | 0.0 | 0.0 | 1.9 | 0.0 |
| V.39 | L.C. | + | " | " " " | 1.9 | 1.9 | 1.8 | 1.7 | 0.1 | 0.2 | 0.9 | 1.6 |
| V.43 | " | + | Clear. | Unchanged. | 2.1 | 1.1 | 1.5 | 1.1 | 0.0 | 0.0 | 1.9 | 0.3 |
| V.45 | " | + | " | Coagulated on boiling. | 2.4 | 2.3 | 2.1 | 1.9 | 0.1 | 0.0 | 1.3 | 1.5 |
| V.47 | M.C. | + | Turbid. | Unchanged. | 2.6 | 1.2 | 1.4 | 1.0 | 0.0 | 0.0 | 0.8 | 0.0 |
| V.13 | " | + | " | Firmly coagulated. | 4.2 | 3.4 | 3.6 | 3.8 | 0.0 | 0.1 | 0.1 | 3.6 |
| V.14 | L.C. | + | " | " " | 3.9 | 3.4 | 3.6 | 3.3 | 0.0 | 0.1 | 0.1 | 3.5 |
| V.16 | " | + | Clear. | Acid 0.7 per cent. | 2.1 | 2.1 | 1.9 | 2.2 | 0.1 | 0.0 | 0.0 | 1.8 |
| V.21 | Pairs and S.C. | + | Turbid. | Partially coagulated on boiling. | 1.9 | 1.6 | 1.8 | 2.0 | 0.0 | 0.0 | 0.1 | 2.1 |
| V.23 | M.C. | + | " | Partially coagulated on boiling. | 2.7 | 2.1 | 1.7 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| V.29 | " | + | " | Acid 0.9 per cent. | 2.8 | 2.8 | 2.1 | 2.4 | 0.0 | 0.0 | 0.0 | 0.0 |
| V.31 | L.C. | + | Clear. | Coagulated on boiling. | 3.4 | 3.5 | 3.3 | 3.5 | 0.0 | 0.0 | 0.0 | 2.5 |
| V.32 | M.C. | + | " | Acid 1 per cent. | 3.4 | 3.0 | 3.3 | 2.8 | 0.0 | 0.0 | 0.0 | 2.2 |
| V.41 | L.C. | + | " | Unchanged. | 1.1 | 1.1 | 1.1 | 1.8 | 0.3 | 0.3 | 0.1 | 0.2 |
| V.44 | M.C. | + | Turbid. | Coagulated on boiling. | 3.0 | 4.1 | 4.0 | 3.7 | 0.0 | 0.0 | 0.1 | 3.3 |
| V.49 | L.C. | + | " | Unchanged. | 2.4 | 2.4 | 2.3 | 2.4 | 0.0 | 0.0 | 0.0 | 1.1 |
| V.6 | M.C. | + | Clear. | Acid 0.7 per cent. | 2.0 | 1.5 | 2.0 | 1.3 | 0.0 | 0.8 | 0.0 | 2.3 |
| V.18 | L.C. | + | " | " 0.8 " " | 2.2 | 2.1 | 1.9 | 2.2 | 0.0 | 0.6 | 0.1 | 1.7 |

TABLE VI—*Concluded.*

| No. of culture. | Grouping. | Gram's stain. | Growth in bouillon. | Milk. | Production of acid in. | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Dextrose. | Lactose. | Saccharose. | Maltose. | Raffinose. | Inulin. | Mannite. | Salicin. |
| | | | | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| V.42 | Pairs and S.C. | + | Turbid. | Coagulated on boiling. | 3.6 | 2.8 | 3.7 | 3.4 | 1.3 | 0.1 | 2.6 | 3.2 |
| V.46 | L.C. | + | Clear. | Unchanged. | 2.3 | 1.2 | 1.5 | 1.8 | 1.1 | 0.0 | 1.0 | 0.8 |
| V.8 | Pairs and S.C. | + | Turbid. | Coagulated on boiling. | 5.4 | 4.2 | 5.4 | 5.7 | 5.3 | 5.7 | 0.0 | 4.9 |
| V.25B. | M.C. | + | Clear. | " " " | 2.0 | 2.6 | 2.3 | 1.9 | 1.8 | 0.0 | 0.0 | 2.3 |

cases of mastitis. Careful examination of the characters of this group, however, reveals distinct differences in many instances. Strains V. 16, V. 21, V. 25B., and V. 49 are low in acid production in the substances which they ferment; in addition, they fail to coagulate milk or produce only sufficient acidity to clot it after boiling. Culture V.29 corresponds somewhat closely with the non-salicin-fermenting type of mastitis streptococci but fails to coagulate milk. The acidity produced by Culture V. 41 is extremely low. In the main the surface colonies on horse blood agar plates have differed from those produced by mastitis streptococci. Vaginal streptococci as a rule produce very tiny, delicate, raised, almost transparent colonies which rarely reach a diameter of 1 mm. after 48 hours incubation. Exceptions, however, are noted in the case of Strains V. 13, V. 14, V. 29, and V. 31. Here the colonies were larger and a trifle more opaque and flattened. Cultures V. 13, V. 14, V. 31, V. 32, and V. 44 possess many characters in common with the streptococci usually associated with mastitis.

As a further means of identification it was decided to test agglutination affinities of the vaginal strains more nearly approaching the mastitis type. The results are shown in Table VII.

The characters of the other six strains of streptococci recorded in Table VI differ to such an extent from those previously isolated from inflamed udders that it seemed useless to consider them further.

Of the 34 vaginal strains two (Strains V.13 and V.14) possessed morphological and cultural characters as well as agglutination affinities in common with the non-hemolytic streptococci associated with mastitis.

TABLE VII.

*Agglutination Titer of the Streptococci Isolated from the Vagina.*

| No. of culture. | Dilution of serum. | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1:100 | 1:500 | 1:1,000 | 1:2,000 | 1:5,000 | 1:10,000 | 1:20,000 |
| Mastitis streptococcus used in immunization. | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| V. 13 | +++ | +++ | +++ | +++ | ++ | + | − |
| V. 14 | +++ | +++ | +++ | +++ | + | − | − |
| V. 21 | − | − | − | − | − | − | − |
| V. 29 | − | − | − | − | − | − | − |
| V. 31 | − | − | − | − | − | − | − |
| V. 41 | − | − | − | − | − | − | − |
| V. 44 | − | − | − | − | − | − | − |
| V. 49 | − | − | − | − | − | − | − |

### *Transmission of the Virus.*

It has been difficult to trace the mode of infection. Table I is indicative that infection may take place directly from animal to animal. Cows 7 and 8 both carried hemolytic streptococci, but Cow 6, a neighbor of Cow 7, was infected with the non-hemolytic type. Cow 9 failed to harbor streptococci and Cow 10, which developed mastitis while under observation, eliminated the hemolytic type. Her neighbor, Cow 11, carried a pure culture of non-hemolytic streptococci. It will be observed that the infections traceable to contact are in the minority. This is especially true of Cows 33, 37, 41, and 50. Two other methods of transfer aside from contamination with vaginal discharges remain: (1) transmission of the specific organisms on the hands of the milker and (2) on the currycombs, brushes, cloths, etc., used in washing the cows.

The "gang" system of milking is employed. Each man is assigned a cow; after milking he washes his hands with soap and cold water and dries them on a fresh towel. He is then assigned to another

cow. In this way one man milks one, two, or three cows irregularly spaced in each barn. Could it be shown that the milker really transmitted the virus on his hands, the irregular occurrence of infections would be readily explained.

The following experiment was devised to test this point. A milker who was known to soil his hands with milk during the process of milking was assigned to milk a cow (No. 10, Table I) known to be eliminating hemolytic streptococci. Before milking, his hands were scrubbed with soap and water and disinfected for 2 minutes in 1:1,000 solution of mercuric chloride. After again washing in water and drying on a clean towel, sterile salt solution was poured over the palms and backs of the hands and collected in a sterile bottle. Salt solution suspensions were prepared with scrapings from beneath the finger nails. His hands were then dried on a fresh towel. After milking, the palms were again rinsed with salt solution. The washings were collected in a sterile bottle. Material from beneath the finger nails was suspended in sterile salt solution. The milker was then instructed to wash his hands in the usual manner (soap and cold water) and dry them. Final washings with salt solution were then collected and scrapings made from the finger nails. The washings and suspensions were brought to the laboratory and plate cultures prepared at once.

*Series A.*—Plate cultures from washings of hands before milking failed to contain hemolytic streptococci.

*Series AA.*—Plates prepared from suspensions of nail scrapings before milking failed to contain hemolytic streptococci.

*Series B.*—Plates prepared from washings of hands after milking contained hemolytic streptococci.

*Series BB.*—Plates prepared from suspensions of nail scrapings after milking contained hemolytic streptococci.

*Series C.*—Plates prepared from washings of hands after milking, washing, and drying hands, contained hemolytic streptococci.

*Series CC.*—Plates prepared from suspensions of nail scrapings after milking, washing, and drying hands did not contain hemolytic streptococci.

To prove definitely that the streptococci found on the hands of the milker were identical with those in the udder, subcultures were inoculated into various media, incubated, and titrated. They were

tested with their specific group serum. The results are given in Tables VIII and IX.

The experiment seems to show that a careless milker may easily carry the virus from an infected to a non-infected cow. This evidence

TABLE VIII.

*Morphological and Biological Characters of the Hemolytic Streptococci Isolated from the Udder and from the Hands of the Milker.*

| No. of culture. | Grouping. | Gram's stain. | Growth in bouillon. | Milk. | Production of acid in. | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Dextrose. | Lactose. | Saccharose. | Maltose. | Raffinose. | Inulin. | Mannite. | Salicin. |
| | | | | | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| E.7* | L.C. | + | Clear. | Firmly coagulated. | 5.0 | 4.2 | 4.3 | 4.2 | 0.1 | 0.1 | 0.1 | 2.3 |
| B.† | " | + | " | " " | 5.0 | 3.9 | 4.5 | 4.1 | 0.1 | 0.1 | 0.0 | 2.2 |
| B.B.‡ | " | + | " | " " | 4.6 | 4.0 | 4.4 | 3.9 | 0.0 | 0.0 | 0.0 | 2.2 |
| C.§ | " | + | " | " " | 4.8 | 4.7 | 4.4 | 4.0 | 0.1 | 0.1 | 0.1 | 2.4 |

* Strain isolated from the udder.

† Strain isolated from the hands of the milker after milking.

‡ Strain isolated from beneath the finger nails of the milker after milking.

§ Strain isolated from the hands of the milker after they had been washed with soap and cold water.

TABLE IX.

*Agglutination Titer of the Streptococci Described in Table VIII When Tested with the Hemolytic Group Serum.*

| No. of culture. | Dilution of serum. | | | | |
|---|---|---|---|---|---|
| | 1:100 | 1:200 | 1:500 | 1:1,000 | 1:2,000 |
| E.7 | +++ | +++ | +++ | ++ | + |
| B. | +++ | +++ | +++ | + | + |
| B.B. | +++ | +++ | +++ | ++ | + |
| C. | +++ | +++ | +++ | + | + |

explains the extreme irregularity of the occurrence of infections. Washing the hands with soap and cold water will not rid them of the virus. A similar experiment was attempted with the non-hemolytic streptococci. They were recovered even after washing. They are

more difficult to recover from the plates because the deeper colonies fail to reveal the more definite characters of the hemolytic types.

Experimental evidence covering infections transmitted by the utensils, water, and cloths used in washing the cows is not available. It seems that if this were the usual method of transmission three or four neighboring animals would be harboring streptococci of the same type. Such has not been frequent.

## DISCUSSION.

Infection of the hind quarters of the udder with streptococci has been more frequent than of the fore quarters. In a number of instances the disease appeared immediately after parturition. This was particularly observed in infections associated with the hemolytic types. The evidence was suggestive of infection of genital origin. Bacteriological examination of the vagina of 64 cows revealed, however, non-hemolytic streptococci identical with those associated with mastitis in only two instances. Hemolytic streptococci have not been isolated from the vagina in a single instance. It must be borne in mind, however, that this may not hold true in other herds. All the animals in this study cannot be regarded as normal individuals. A mucopurulent discharge from the vagina was observed in a considerable number of cows. Many had aborted. Others were suffering from vaginitis or metritis. Retention of the placenta had occurred in several instances.

One point which may be of value to those concerned in the study of public health problems is the number of apparently normal cows actually shedding streptococci in the milk. In previous papers it has been shown that these organisms may gain access to the milk supply. It was not known to what extent this held true, but the milk from sixteen cows among 50 in one barn contained streptococci. Nine were harboring the hemolytic type. While the number of cows eliminating such organisms in the milk may not be so high in the average as in this herd, nevertheless, they must exist to a considerable degree. The number of infected samples from this barn is sufficient to enable one to isolate streptococci from samples of the whole supply. The milk is sold as raw milk. If the bovine

streptococci associated with mastitis and identical with them were capable of causing tonsillitis in persons consuming the milk from this dairy, these conditions would have aroused attention and caused an investigation of the milk supply. Such has not been the case.

It must be remembered that in the herd from which a large proportion of the material was obtained the incidence of mastitis was high. In herds where the incidence is low the probable number of carriers would be proportionately low.

## CONCLUSIONS.

1. The principal sources of streptococcic infection, aside from clinical cases, are apparently normal cows which carry the virus in the udder.

2. These carriers may be grouped as follows: (*a*) those that have been infected recently and have not yet developed symptoms; (*b*) those that have suffered from inflammation of the udder and after recovery still harbor streptococci; (*c*) those that have had no clinical history of mastitis. There is some evidence to lead one to regard the latter group as naturally immune.

3. A milker may readily carry streptococci on his hands from an infected to an uninfected cow.

4. The vaginæ of 34 of the 64 cows examined contained nonhemolytic streptococci. Of the 34 strains isolated 32 differed in their cultural characters and agglutination affinities from those associated with mastitis. The other two strains may be regarded as of etiological significance.

5. In no instance have hemolytic streptococci been isolated from the vagina.

# THE SURVIVAL OF THE HOG-CHOLERA VIRUS IN LABORATORY ANIMALS, PARTICULARLY THE RAT.

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In beginning an investigation of the filterable virus of hog-cholera one line of work decided upon was to study the effect of the virus on various laboratory animals in the hope that some reaction or local infection might be produced that previously had been overlooked. Although Uhlenhuth and Haendel (1) state that the mouse, rat, guinea pig, rabbit, dog, cat, horse, donkey, sheep, cow, goat, pigeon, chicken, goose, and duck are not susceptible to inoculation with the virus, it was nevertheless hoped that by using some of the less common methods of inoculation and by carefully observing the animals some species other than swine might be made available for work on hog-cholera. The method chosen was to inoculate several animals of a given species in one or more ways, to observe them closely, and after approximately 7 days to determine whether the virus was still present in their bodies by inoculation of susceptible pigs.

## HISTORICAL.

Craig (2) has reported work along the same line. He injected large amounts of virus (15 cc.) intravenously into rabbits and 8 days later made a suspension of their organs and tissues, and, after passing it through a Berkefeld filter, injected it into pigs. In several instances the animals came down with hog-cholera, showing that the virus had remained alive for 8 days in the body of the rabbit. Attempts to pass the virus from one rabbit to another were negative.

Sir Stewart Stockman (3) attempted to infect wild rats with hog-cholera by feeding them the intestines of pigs showing lesions of the disease. Two lots of rats were fed daily, one for 12 and the other for 11 days, after which they were killed and as much blood as possible was obtained from the heart. The temperature of the pig inoculated with the blood from the first lot rose to between 104° and 105°F. on the 6th day after the injection and remained there for 3 days, after

which it returned to normal. The animal was killed 16 days after the inoculation and no lesions of hog-cholera were found. The pig inoculated with the blood from the second lot of rats showed no temperature or other signs of illness for 42 days following the injection. The result of the inoculation of the first pig must be looked upon as doubtful, but even though it were negative the conclusion that could be drawn would be that the rats did not have the virus in their blood stream and not "that rats are not, as has been suggested, pathological carriers of swine-fever," for the virus might be carried in some organ in the body and eliminated with the urine or feces and still not be present in the blood.

## EXPERIMENTAL.

*Experiment 1.*—Object: To determine whether after inoculation with hog-cholera virus rabbits will show a febrile or other reaction and to determine whether the virus is destroyed in their bodies in 12 days.

A rabbit weighing 1,038 gm. was inoculated intravenously with 1 cc. of sterile, but unfiltered, serum that was proved, by inoculation into a pig, to contain the virus of hog-cholera. On the same day a rabbit weighing 970 gm. was inoculated intraperitoneally with 1 cc. of the same serum. A third rabbit weighing 1,815 gm. received into the testicle 1.4 cc. of the same virus.

The temperature of these rabbits was normal for the next 10 days and there were no visible signs of illness. The rabbit injected into the testicle showed no swelling or local lesion in this organ.

12 days after inoculation the first two rabbits were killed and their abdominal and thoracic viscera and brains fed to a pig weighing 94 pounds. The temperature of this pig was normal for the next 17 days and later it was shown to be susceptible to hog-cholera virus.

*Experiment 2.*—Will the virus live in the body of the guinea pig 6 days?

A guinea pig weighing 495 gm. was given an intraperitoneal injection of 5 cc. of virulent virus. The animal immediately showed anaphylactic symptoms and its temperature fell to 35.4°C. Another experiment showed that the serum from a normal pig will produce the same effect in guinea pigs. Several hours later the temperature of this animal was normal and it remained so for the next 6 days. It was then chloroformed and a suspension made of pieces of its liver, kidney, lungs, and heart. The spleen was contaminated and was not included. 5 cc. of this thick suspension were injected intramuscularly into a pig weighing 48 pounds. The animal showed no temperature reaction and gained 12 pounds in the next 14 days. It was then inoculated with a virus of very low virulence and showed no reaction. Later it was proved to be immune by inoculation with an active virus and by exposure. I am inclined to believe that the inoculation with the virus of low virulence gave this pig an immunity, but the fact that it was immune makes the interpretation of the experiment doubtful.

*Experiment 3.*—Object: To determine whether the virus of hog-cholera can be demonstrated in the bodies of pigeons 7 days after inoculation.

Two pigeons were each given an intravenous injection of 1 cc. of active virus and two others were each given an intracerebral injection of ½ cc. of the same virus. During the next 7 days their temperatures were slightly more irregular than those of two uninoculated pigeons kept with them. All the pigeons appeared well and the inoculated ones were chloroformed in 7 days and suspensions made from their livers, spleens, kidneys, hearts, lungs, brains, and pieces of their breast muscle. These organs and tissues were removed, using aseptic precautions, and the suspension was not filtered but was injected directly into a pig weighing 33 pounds. The animal showed no signs of illness for the next 25 days when it was inoculated with virulent virus to which it succumbed. The experiment shows that intravenous and intracerebral injections of hog-cholera virus into pigeons failed to produce disease and 7 days after inoculation the virus could not be demonstrated in their organs and tissues.

*Experiment 4.*—Four half grown white rats were inoculated with active, sterile, but not filtered, serum from a pig with acute hog-cholera. Two of the rats were given intraabdominal injections of 1 cc. and the other two intracerebral injections of 0.5 cc. of the serum. One of the latter rats died soon after the injection so it will not be considered further. The rats showed no effects from the inoculation and were chloroformed at the end of 7 days. At autopsy no abnormalities were noted. The lungs, hearts, spleens, kidneys, brains, and portions of the livers and muscles of the thigh were passed through a tissue grinder and suspended in an equal amount of sterile salt solution and infused for 5 hours. At the end of this time 9 cc. were injected intramuscularly into the thigh of a susceptible pig weighing 25 pounds. The temperature of this animal was normal for 3 days after which it went up to 40.3°C. on the 4th, 40.5°C. on the 5th, and 40.9°C. on the 6th day following the inoculation. The temperature then fell for 2 days after which it went to 41.5°C. During this time the pig was very sick. It was killed on the 10th day following the injection and showed moderately congested lymph nodes, hemorrhages under the capsule of the kidney, and small ulcers around the ileocecal valve. Cultures from the liver, spleen, and kidney showed no growth. The bacteria-free serum when injected into a susceptible pig produced hog-cholera. We may conclude from this experiment that virulent hog-cholera virus remains in the body of the white rat for at least 7 days.

*Experiment 5.*—Object: To confirm the results of Experiment 4 and to determine whether both types of injection are effective in keeping the virus in the rat's body.

Four rats weighing between 120 and 144 gm. were selected and two of these were each given an intraperitoneal injection of 1 cc. of the serum of the pig used in Experiment 4. The other two were each given an intracerebral injection of 0.5 cc. of the same serum. 7 days after inoculation the two rats that received intraabdominal injections were chloroformed and their livers, spleens, kidneys, testicles, lungs, hearts, and brains passed through a tissue grinder and a 20 per cent suspension was made in salt solution. 10 cc. of this suspension, which represented 2 gm. of tissue, produced typical acute hog-cholera when injected into

a susceptible pig. After sterilizing the instruments and grinder the two rats which had received the intracerebral inoculation were chloroformed, the same organs and tissues as in the other rats were passed through the grinder, and a 20 per cent suspension was made in salt solution. 10 cc. of this suspension also produced typical acute hog-cholera in a susceptible pig. We may conclude from this experiment that the virus of hog-cholera may be demonstrated 7 days after its injection into white rats, whether the injection is intraabdominal or intracerebral.

*Experiment 6.*—Object: To determine whether the virus is present in the rat 10 days after either intraperitoneal or intracerebral injection.

Two rats were each given an intraperitoneal injection of 1 cc. of the same virus used in Experiment 5, and two other rats were each given an intracerebral injection of 0.5 cc. of the same virus. They were chloroformed after 10 days and a 10 per cent suspension of their organs and tissues was made according to the method used in Experiment 5. The injection of 10 cc. of both of these suspensions into susceptible pigs produced no effect in the month's time that the animals were under observation. The susceptibility of the pigs was not tested but the other animals in the litter were susceptible, so we may conclude that in this experiment the virus of hog-cholera failed to live in the body of the white rat for 10 days after either intraperitoneal or intracerebral injection.

All the above experiments were made with a single strain of virus that had been obtained from Dr. V. A. Moore. It had been passed from pig to pig by inoculation for many generations and it is conceivable that as a result it had been modified so that it was not so susceptible to destructive action of the rat tissues as would be the natural virus.

*Experiment 8.*—Object: To determine whether another strain of virus recently obtained from the field would live in the rat for 7 days and also to determine in which of the abdominal organs it can be found.

Two white rats were each given an intraperitoneal injection of 1 cc. of a virus two generations removed from the natural strain; that is, the virus had been passed through two of our swine. The rats were chloroformed 7 days after the injection and with separate sterile forceps the spleens, kidneys, and portions of the livers were removed and transferred to weighed Petri dishes. The weights of the organs having been determined, they were ground with sterile sand and 5 per cent suspensions made in salt solution. After standing at room temperature for 3 hours they were injected into pigs. The data for this part of the experiment and the results are given in Table I.

The experiment indicates that 7 days after an injection into the peritoneal cavity of the white rat the virus of hog-cholera is present

in the spleen, it is not present in the liver, and the results of the experiment in as far as the kidneys are concerned are uncertain. It is probable that the pig injected with the kidney suspension suffered from a mild hog-cholera which made it immune. The experiment also shows that this nearly natural strain of hog-cholera virus will live in the rat for at least 7 days.

The above experiments clearly demonstrate that the virus of hog-cholera will live in the body of the white rat for at least 7 days. Rats are constantly associated with swine and it is possible that they

TABLE I.

| Pig. | | Intramuscular injection of 5 cc. of 5 per cent suspension of rat. | Result. |
|---|---|---|---|
| No. | Weight. | | |
| | *lbs.* | | |
| A | 28½ | Spleen. | Temperature up on 4th day. Killed 16 days after inoculation. Kidney hemorrhagic. Ulcers in colon. Filtered urine and serum produced hog-cholera in other swine. |
| B | 28 | Liver. | No effects from inoculation. Exposed to Swine A 9 days after inoculation, as a result contracting acute hog-cholera. |
| C | 31 | Kidneys. | Temperature slightly above normal on the 5th day after the inoculation but the animal did not appear sick. Subsequent inoculation with filtered urine from typical case of hog-cholera and exposure to pigs with hog-cholera were negative. Killed. No evidences of an old hog-cholera infection found. |

may act as intermediate hosts, so the following experiments were made to determine whether or not they could be infected in a more natural way than by injection.

*Experiment 9.*—Four white rats were fed muscle from a pig that was killed 10 days after it had been injected with virus and while it was moribund. 8 days after the feeding of this infected material two of the rats were chloroformed and a suspension of their spleens, kidneys, hearts, lungs, and portions of their livers was injected into a pig weighing 30 pounds. The animal was under observation 35 days during which time its temperature and general appearance were normal. At the end of this period it received a test inoculation with hog-cholera virus and after 10 days it died, showing on autopsy typical hog-cholera lesions.

The other two rats were allowed to live a month, during which time they appeared normal and when chloroformed and autopsied no abnormalities were found.

In this experiment we were unable to demonstrate the virus of hog-cholera in the bodies of white rats 8 days after they had been fed virus in the shape of meat from a diseased pig.

*Experiment 10.*—Object: To repeat the attempt to infect rats by feeding material containing hog-cholera virus and in addition to determine whether the virus could be demonstrated in their urine.

Two rats, one weighing 108 and the other 145 gm., were allowed to fast for several hours and then fed pieces of spleen and kidney from a pig dying of hog-cholera. 2 days later they were transferred to an improvised metabolism cage arranged in such a way that their feces were caught in a funnel and their urine was filtered through into a sterile test-tube. Urine was collected on the morning of the 3rd, 5th, and 6th days after the inoculation and placed at once in a refrigerator at approximately 7+° C. The total amount of urine collected from the two rats was about 7 cc. and as this was contaminated with feces it contained many bacteria, but the amount was so small that it did not seem wise to attempt to filter it. The next day after the major portion of urine was collected the whole amount was injected subcutaneously into a pig weighing 51 pounds. The only result of the inoculation was a large abscess at the site of the inoculation. 13 days after the urine was injected the pig was given an intramuscular injection of hog-cholera virus which produced a typical attack with lesions characteristic of hog-cholera.

The rats fed with the virus were killed after 7 days and a 5 per cent suspension was made of their spleens of which 5 cc. were injected intramuscularly into a pig weighing 36 pounds. The animal showed no effects from the inoculation and 15 days later it was given an intramuscular injection of hog-cholera virus to which it succumbed.

In this experiment the attempt to infect white rats by feeding material containing the hog-cholera virus was unsuccessful, as was the attempt to demonstrate the virus in the urine and feces of these same rats.

*Experiment 11.*—Object: To determine whether a combined infection of hog-cholera virus and hog-cholera bacilli produces in the rat a condition that differs from that caused by the injection of either of these viruses alone.

We have found that at least one of our cultures of the hog-cholera bacillus will produce in the rat a febrile disease either by feeding or by subcutaneous injection. The bacillus can be found in the spleens of these animals at least 21 days after inoculation.

Six rats were weighed, marked, and allowed to fast for 6 hours and then fed meat on which was sprinkled a 24 hour bouillon culture of Hog-cholera Bacillus XII. The next day the rats were divided into lots of three each and the individuals of one lot were each given an intraabdominal injection of 1 cc. of hog-cholera virus that later was proved to be virulent. In addition three uninoculated rats were each given an intraperitoneal injection of the same virus.

The average weights of the three groups of rats show that the gain in weight of the rats that were fed the hog-cholera bacilli was not so great as that in the rats injected with the virus alone, but no visible illness was caused by the combined infection of virus and bacilli.

7 days after the injection of the virus the rats were killed and their abdominal and thoracic viscera fed to pigs weighing about 40 pounds. The animal given the viscera of the rats fed hog-cholera bacilli and injected with hog-cholera virus showed a slight rise in temperature, beginning the 2nd day after the feeding and continuing for 4 days. During this time it was eating and acting normally and for the next 7 days it seemed to be normal in every way. It was then injected with hog-cholera virus to test its susceptibility and died on the 10th day following the inoculation. On autopsy typical lesions of hog-cholera were found and the hog-cholera bacillus was isolated in pure culture from the liver and spleen.

The pig fed the viscera of the rats injected with the virus alone showed no temperature or other evidences of illness during the next 10 days and during this time it gained 15 pounds. It was inoculated with hog-cholera virus to test its susceptibility and 8 days later was chloroformed when it was very sick. Autopsy showed typical lesions of hog-cholera. Cultures were negative.

The experiment shows that a combined infection of hog-cholera bacilli and virus in the white rat caused no more evidences of disease than did the bacilli alone. The attempts to infect pigs by feeding them the viscera of rats that had been injected with hog-cholera virus were negative.

*Experiment 12.*—Object: To determine whether the virus could be passed from one rat to another.

Three rats weighing between 102 and 113 gm. were each given an intraperitoneal injection of 1 cc. of a serum that later was proved to be active. 7 days after the inoculation the rats were chloroformed and a 10 per cent suspension was made of their ground livers, spleens, and kidneys. 1 cc. of this suspension was injected intraperitoneally into each of three rats weighing between 95 and 98 gm. Several days later these rats were chloroformed and a 10 per cent suspension was made of their livers, spleens, and kidneys. 5 cc. of this suspension were injected intramuscularly into a pig weighing 31 pounds. This pig was apparently normal for the next 24 days. It was then inoculated with virulent virus to which it promptly succumbed, showing that the virus was not present in the second series of rats.

*Effect of the Virus of Hog-Cholera on the Rat.*

During these experiments the rats inoculated with the virus have been observed carefully and frequently compared with uninoculated animals kept with them. The inoculated rats do not look sick and they gain in weight the same as the controls. Their temperatures show marked fluctuations but this is true of the normal animals and no differences between the two have been detected. With sera and spleen suspensions from inoculated rats and albumin-free but virus-containing urine from hog-cholera pigs as antigen, precipitin and complement fixation reactions have been negative. In other words, the virus apparently produces no disease in the rats.

## DISCUSSION AND CONCLUSIONS.

The attempts to demonstrate the virus of hog-cholera in rabbits 12 days after intravenous and intraabdominal inoculations were unsuccessful. Likewise the attempts to show that the virus might be found in the guinea pig 6 and in the pigeon 7 days after inoculation were negative. It was shown, however, that the virus can be found in the bodies of white rats for at least 7 days after either intraabdominal or intracerebral inoculations. One attempt to demonstrate it after 10 days was negative. From the fact that the rats show no evidence of illness such as loss in weight, pyrexia, or visible pathological changes, and that after either intraabdominal or intracerebral inoculation the virus is only found in the abdominal organs and possibly only in the spleen, it seems likely that it does not multiply but that in the rat tissue, particularly in the spleen, it is not destroyed so rapidly as in the organs of other animals. Careful study of the records fails to show that passing one strain of virus alternately through pigs and rats for three transfers in each species changes the virulence for swine or causes the virus to become virulent for rats.

Attempts to introduce the virus into the body of the rat by feeding virulent material and an attempt to pass the virus from one lot of rats to another were unsuccessful, so that we have evidence from the experiment that the rat does not play a part in the transmission of hog-cholera.

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# A STUDY OF PARATYPHOID BACILLI ISOLATED FROM CASES OF HOG-CHOLERA.

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Many swine infected with hog-cholera undoubtedly find their way into our food supply and it would seem that a thorough knowledge of the bacteriology of the disease is of importance from a practical as well as from a theoretical point of view. In the earlier days of the work on this disease attention was chiefly confined to the identification of the hog-cholera and swine-plague bacilli, while since the discovery that a filterable virus is the cause of the condition the bacteriology has to a considerable extent been poorly done or neglected altogether. These so called secondary invaders probably vary with the locality from which the animals are obtained, and also with the virulence and possibly other factors associated with the virus. Accordingly the findings I have to report are a contribution to the bacteriology of the disease rather than a bacteriological study, for only two strains of virus have been used and the majority of the animals came from a single herd. *Bacillus coli*, *pyocyaneus*, *alkaligenes*, several species of anaerobes, and several species of unidentified aerobes have been isolated, but this report will be confined to a description of the paratyphoid bacilli found, because of their economic importance, in that they may be the cause of disease in man, and because they bring up some interesting points in the classification of this group.

## HISTORICAL.

The classification of the paratyphoid group is extremely difficult and confusing, for, until recently, there have been no cultural differences between members of the group that could be relied upon, cross-agglutination reactions are common, and animal inoculations have not been made. Dr. Smith (1) stated that the only reliable means of differentiating the hog-cholera bacillus from closely related forms

was by inoculation into rabbits. The former caused a definite disease after subcutaneous injection, resulting in death in from 7 to 12 days with marked changes in the lymphoid tissue, and in freshly isolated cultures with necroses in the liver. The failure to make rabbit inoculations probably accounts for the failure to differentiate the hog-cholera bacillus from paratyphoid $\beta$ in spite of the statement of Uhlenhuth, Hübener, Xylander, and Bohtz (2) that such inoculations are of no value in differentiating the two.

Dr. Smith noted that the hog-cholera bacillus grew less readily in bouillon than did typhoid or other paratyphoids. Ford (3) suggested that the hog-cholera bacillus could be differentiated from the other members of the group by its failure to ferment arabinose. Jordan (4) and Krumwiede, Kohn, and Valentine (5) have recently shown that the hog-cholera bacillus cultures which they have studied failed to ferment arabinose and in addition that dulcite was fermented little or not at all, and the latter authors have extended the observations of Weiss and Rice (6) on inosite and they show that paratyphoid $\beta$ is the only member of the group that ferments this carbohydrate. Jordon and Victorson (7) show that the hog-cholera bacillus differs from paratyphoid $\beta$ in that it fails to form hydrogen sulfide in peptone agar containing lead acetate, whereas the latter and *B. enteritidis* promptly cause a blackening of the medium. A reaction that is somewhat similar is noted by Krumwiede, Kohn, and Valentine (5). They find that in a serum water containing 0.1 per cent dextrose and 1 per cent of Andrade indicator paratyphoid $\alpha$ and the hog-cholera bacillus fail to reduce the fuchsin while paratyphoid $\beta$ and *enteritidis* give a colorless or at most a faintly pink coagulum at the end of 24 hours incubation.

By means of the agglutination reaction Uhlenhuth, Hübener, Xylander, and Bohtz (2), Savage (8), Jordan (4), and Krumwiede, Kohn, and Valentine (5) agree that paratyphoid $\alpha$ and $\beta$ and *enteritidis* can be differentiated from one another, but the first authors state that paratyphoid $\beta$ and hog-cholera bacillus cannot be differentiated by this reaction, while the others say that they can, especially when absorption tests are made.

When one examines the literature of the paratyphoid bacteria, it is found that most workers have considered all members of this group to be hog-cholera bacilli, no matter whether they came from normal or diseased swine. Grabert (9) is often cited as having isolated hog-cholera bacilli from normal swine but the only carbohydrates he used were dextrose, lactose, and saccharose, and in immunizing rabbits two animals survived the injection of 0.01 cc. of a bouillon culture intravenously, which goes a great way towards showing that he did not have typical hog-cholera bacilli. Seiffert (10) compared three strains of what he calls hog-cholera bacilli with paratyphoid $\beta$ and found that they were the same. It should be noticed, however, that he made no tests for pathogenicity and two of the strains formed acid and gas from arabinose, while the other was not tested, and all of them formed hydrogen sulfide. Not one of the tests used by Uhlenhuth, Hübener, Xylander, and Bohtz would differentiate

the hog-cholera bacilli from other paratyphoids, and as they did not make rabbit inoculations it is impossible to say whether they were working with the true hog-cholera bacillus or with another member of the group. Krumwiede, Kohn, and Valentine (5) seem to think that the hog-cholera bacilli are the only paratyphoids found in swine, for, after showing that paratyphoid $\beta$ and the hog-cholera bacillus can be separated both culturally and serologically, they conclude that the former is found only in man.

## EXPERIMENTAL.

As the cultures to be described came from swine infected with the virus of hog-cholera it seemed wise to repeat the newer cultural tests for the hog-cholera bacillus. Six strains, all of which had been studied by Dr. Smith and pronounced true hog-cholera bacilli, were used and it was found that all six failed to ferment arabinose in 10 days and all but one failed to ferment dulcite in the same time. This one culture which did ferment dulcite failed to do so after it had been passed through a pig, showing how unstable this property is. All the cultures failed to produce a brown color in peptone-lead acetate agar and all gave a decidedly pink color in Krumwiede's dextrose-Andrade-serum water.

The cultures to be described were isolated during the course of some experimental work on hog-cholera in which two strains of virus were used. One of the strains came from Dr. V. A. Moore, while the other was obtained from a spontaneous outbreak in New Jersey. During the work thirty-eight pigs were used, weighing between 20 and 45 pounds. The majority of the animals were raised on the Institute farm where their parents had been isolated from other swine for 3 years. Thirty-three of the pigs were infected by exposure or by the inoculation of bacteria-free material containing the virus, while the remaining five were spontaneous cases obtained from nearby places. Most of the animals were killed in from 7 to 12 days after their temperature began to rise, while some were allowed to die. All showed characteristic lesions of hog-cholera and the sera of many of them were used to infect other pigs.

Cultures were made as a routine from the liver, spleen, and kidney, after searing the surface of the organs, by digging out bits of tissue with flamed forceps and transferring these to agar slants and often-

TABLE I.

*Source of Cultures.*

| Culture No. | From. | | History of swine inoculated. | | | Disposed of. |
|---|---|---|---|---|---|---|
| | Swine No.* | Organ. | Date. | Weight. | Material. | |
| | | | *1917* | *lbs.* | | |
| Swine-typhus I | 149 | Liver; spleen; kidney. | June 29 | 18 | Fed viscera from spontaneous case of cholera. | July 7. Died. Autopsy 12 hrs. post mortem but animal had been kept cold. Few hemorrhages under capsule of kidney. Fibrinous exudate in cecum. Hemorrhagic pneumonia. |
| Swine-typhus II | 152 | Liver; spleen; kidney; lymph node. | July 20 | | Exposed to spontaneous case. | Aug. 14. Died. Autopsy 5 hrs. post mortem. Typical lesions of hog-cholera. |
| Swine-typhus III | 151 | Liver. | Aug. 1 | 47 | Exposed to Swine 152. | Aug. 30. Moribund. Chloroformed and autopsied at once. Typical lesions of hog-cholera. |
| Swine *enteritidis* I | 161 | Liver; spleen. | " 21 | 40 | " " fresh spontaneous case of cholera. | Sept. 19. Chronic hog-cholera. Killed. Autopsy shows healing lesions of cholera. |
| Swine-typhus IV | 192 | Liver; spleen; kidney. | Dec. 12 | 21 | Inoculated with organ emulsion of rats that had been inoculated with virus 7 days before. | Dec. 19. Sick. Killed. Typical lesions of cholera. |
| | | | *1918* | | | |
| Swine typhus V | 194 | Liver. | Jan. 29 | 25 | Inoculated with filtered emulsion of liver, spleen, and kidney of virus pig. | Feb. 8. Moribund. Chloroformed. Typical lesions of hog-cholera. |

* The numbers used belong to a series that was begun many years ago so that they in no way indicate the number of animals used.

times to fermentation tubes containing bouillon. Where growth occurred pure cultures were obtained and from one or more organs of six of the thirty-eight swine bacteria belonging to the paratyphoid group were isolated.

The numbers given to these cultures and a condensed history of the animals from which they were obtained are given in Table I. It is worthy of note that four of the animals were exposed to spontaneous cases of hog-cholera while the other two were purchased from a neighboring farm before inoculation. The animals to which the first four were exposed did not show paratyphoid bacteria in their organs but they must be looked upon as a possible source of the infection. Of a large number of our own animals inoculated with bacteria-free virus not one has shown paratyphoid bacilli in its organs, indicating that they do not carry these organisms in their gastrointestinal tracts.

*Cultural Characteristics.*

All six cultures resemble one another in that they are motile, Gram-negative rods of about the size of the typhoid bacillus. They grow readily on the ordinary media. On agar they form translucent bluish colonies, while in bouillon they cause a turbidity of about the same density as that caused by the typhoid bacillus. They do not liquefy gelatin or produce indol. They all form acid and gas in fermented bouillon containing 1 per cent dextrose or mannite, while they fail to attack lactose or saccharose. In Durham tubes containing fermented bouillon plus 1 per cent of Andrade indicator and 1 per cent xylose, dulcite, or arabinose they form acid and gas in 24 hours from all three carbohydrates, while neither acid nor gas is produced in salicin bouillon in 10 days. In lead acetate-peptone agar they all produce a brownish color in 24 hours, indicating the formation of hydrogen sulfide. In the medium of Krumwiede made of sterile horse serum one part, sterile distilled water four parts, containing 1 per cent of Andrade indicator and 0.1 per cent dextrose, all six cultures produce in 18 hours a practically colorless coagulum containing gas bubbles. In litmus milk they all give a distinctly alkaline reaction in from 3 to 6 days.

By comparing the cultural reactions of these bacteria with those given for the hog-cholera bacillus it will be seen that there are distinct differences, and from the findings of Jordan and Krumwiede already referred to they would have to be classed as paratyphoid $\beta$ or *enteritidis*. These two may be differentiated by the fact that the former ferments inosite, but the one test made with the swine cultures was unsatisfactory and no more of the carbohydrate could be obtained.

*Pathogenicity.*

When injected into the subcutaneous tissues of the rabbit in large numbers these cultures cause a local infection from which the animals recover. The tests are summarized in Table II where it will be seen that 0.1 cc. of a 24 hour bouillon culture failed to kill any of the rabbits.

TABLE II.

*Virulence Tested.*

| Rabbit. | | Subcutaneous injection of 0.1 cc. of a 24 hr. bouillon culture of strain. | |
|---|---|---|---|
| No. | Weight. | Culture No. | Effect. |
| | *gm.* | | |
| 1 | 2,476 | Swine-typhus I | Slight loss in weight with open ulcer at site of inoculation. Recovery. |
| 2 | 1,572 | " I | Slight pyrexia, loss in weight, and local lesion. Recovery. |
| 3 | 2,505 | " II | Slight pyrexia, marked loss in weight, and local lesions. Chloroformed 1 month after inoculation. No lesions found; organs sterile. |
| 4 | 2,740 | " II | Pyrexia, marked loss in weight, and large local lesion. Recovery. |
| 5 | 2,325 | " III | Dead in 6 days. Rabbit septicemia. |
| 6 | 2,168 | " III | Pyrexia, loss in weight, and local lesion. Recovery. |
| 7 | 2,609 | Swine *enteritidis* I | " " " " " " " " |
| 8 | 2,137 | " " I | " " " " " " " " |
| 9 | 2,500 | Swine-typhus IV | Loss in weight and local lesion. Chloroformed 19 days after inoculation. No lesions found and large pieces of liver and spleen were sterile. |
| 10 | 2,226 | " IV | Pyrexia, loss in weight, and local lesion. Recovery. |
| 11 | 1,167 | " V | Amount injected 0.5 cc. Rise in temperature, loss in weight, and large abscess at site of inoculation. Recovery. |

In some cases the local lesions were quite extensive and it is probable that had injections been made into the peritoneal cavity death would have resulted. Such injections did not seem necessary as the object was to differentiate these cultures from the hog-cholera bacillus, for, as has been emphasized above, the latter organism always kills the rabbit when injections of 0.1 cc., or even less, of a 24 hour bouillon culture are made into the subcutis. It should also be noted that two of the animals were killed, one 19 and the other 30 days after the injection and that no lesions were found and cultures made by transferring large bits of liver and spleen to bouillon remained sterile, indicating that there had been no general invasion of the body of the animals.

Four mice were fed a bouillon culture of one of these strains (Swine-typhus III). 6 days later one was killed, and though it was apparently well, cultures from the liver and spleen showed a paratyphoid bacillus that was probably the organism ingested. The remaining three animals were not visibly affected by the feeding.

White rats were inoculated with 0.1 cc. of 24 hour bouillon cultures of four of these strains. They showed a slight loss in weight and a local lesion, but they had apparently recovered at the end of 22 days, when they were killed and cultures made from their livers and spleens. Organisms of the paratyphoid group were obtained from two of these rats.

A pig weighing 37 pounds was fed 50 cc. of a 24 hour bouillon culture of Swine-typhus III mixed with ground food, all of which was eaten. The animal showed no effect from the feeding either in its temperature, its appetite, or the consistency of its feces for the next 8 days. It was then inoculated with virulent hog-cholera virus and killed 9 days later when it was very sick. Autopsy showed the typical lesions of hog-cholera. Cultures made from bits of the liver, spleen, and kidney were sterile, showing either that the bacillus was not present in the digestive tract or, if present, it did not have the power to invade the body as does the hog-cholera bacillus when it is fed to a pig and the animal later is inoculated with virus.

The results of the animal inoculations show that the six cultures are not nearly so pathogenic for rabbits as are the hog-cholera bacilli. My experience with paratyphoid $\beta$ is limited but it seems that these

swine cultures produced larger local lesions than the former does, but this may be due to the fact that the swine cultures have been isolated more recently than the human strains worked with.

*Serological Tests.*

In preparing animals for immune sera the rabbits used in testing the virulence of the different strains of swine cultures were utilized. After they had recovered from the subcutaneous injection of 0.1 cc. of a 24 hour bouillon culture they were given an intravenous injection of 0.01 cc. of a bouillon culture of the same age. 10 days after the last injection they were bled. This method of treatment gave sera of high agglutination titer but it is possible that such sera differ from those produced by the injection of large numbers of bacteria killed by heat. Uhlenhuth and Hübener (11) state that they get better agglutinating sera for the paratyphoid group by immunizing with killed than with living cultures. Though the organisms are motile they say that the type of agglutination is not characteristic and that all gradations between fluffy and fine clumps may be observed.

When tested against sera produced by the injection of living cultures it was found that one of the strains was sharply marked off by the fact that it failed to be agglutinated by the sera of animals immune to the others and the serum of a rabbit immune to it did not agglutinate the others. The data substantiating these statements are found in Table III.

The serum of this rabbit immune to Swine *enteritidis* I was then tested on other paratyphoids from animals and man and it was shown that the culture is one of *Bacillus enteritidis*, for its serum agglutinates other *enteritidis* cultures as well as the majority of the animal cultures. These consist of two mouse, four rat, three guinea pig, and one dog typhus and two human *enteritidis* cultures. Absorption tests show that these animal and human strains will absorb the agglutinins for the swine as well as for the other strains that are agglutinated by this serum.

The other five strains, which for convenience have been called swine-typhus, appear to be the same serologically as well as culturally. They are agglutinated to the same titer limit and any one of them will absorb the agglutinins for the others.

TABLE III.

*Agglutination of Swine Cultures by Sera of Rabbits Immune to Strains That Differ Serologically.*

| Culture tested. | Limit of agglutination with serum of rabbit. | |
|---|---|---|
| | Immune to Swine-typhus III. | Immune to Swine *enteritidis* I. |
| Swine-typhus I | 51,200 | 100—* |
| " II | 51,200 | 100— |
| " III | 51,200 | 80 |
| Swine *enteritidis* I | 1,600 | 51,200 |
| Swine-typhus IV | 51,200 | 100— |
| " V | 12,800+ | 320 |
| Hog-cholera Arkansas | 1,600 | 80— |
| " XII | 1,600 | 160— |

* Figures followed by a minus sign indicate that there was no agglutination in this the lowest dilution used.

### *Relation of Swine-Typhus to Hog-Cholera Bacilli.*

Though cultural differences have been found between these cultures and those of the hog-cholera bacillus, it is interesting to compare the two serologically.

The agglutination relationship to various hog-cholera bacillus cultures is shown in Table IV, where it will be seen that there is a certain amount of cross-agglutination but the limit of clumping is much lower than in the control strains, and in addition the sediment, after the tubes have been incubated 2 hours and refrigerated over night, is firm and compact, whereas with the homologous strains it is loose and fluffy.

Absorption tests show that cultures of Swine-typhus I and II will take from the anti-hog-cholera bacillus serum the agglutinins for themselves and Swine-typhus V, although the agglutinins for the hog-cholera bacilli are practically unchanged, and that the hog-cholera bacilli will take from the Anti-swine-typhus III serum the agglutinins for themselves without removing those for Swine-typhus III. These facts are shown in Table V.

Thus we see that while there is a certain relationship, as shown by

TABLE IV.

*Agglutination Relationship of Hog-Cholera and Swine-Typhus Bacilli.*

| Culture tested. | Limit of agglutination with serum of rabbit. | |
|---|---|---|
| | Immune to Swine-typhus III. | Immune to hog-cholera bacillus. |
| Swine-typhus I | 51,200 | 12,800×* |
| " II | 51,200 | 12,800× |
| " III | 51,200 | 6,400× |
| " IV | 51,200 | 6,400× |
| " V | 12,800+ | 1,600× |
| Hog-cholera bacillus Nebraska | 1,600× | |
| " " Denver | 3,200× | |
| " " Arkansas | 3,200× | 25,600+ |
| " " X | 400× | |
| " " XI | 1,600× | |
| " " XII | 1,600× | 12,800 |

* × indicates that the sediment was firm and compact, +, the highest dilution tested, clumping very marked.

TABLE V.

*Absorption of Agglutinins from Hog-Cholera Bacillus and Swine-Typhus Sera by Swine-Typhus and Hog-Cholera Bacilli Respectively.*

| Serum. | | | Cultures tested with highest dilution in which agglutination occurred. | | | | |
|---|---|---|---|---|---|---|---|
| Rabbit No. | Immune to. | Absorbed with. | Swine-typhus II. | Swine-typhus I. | Swine-typhus V. | Hog-cholera Arkansas. | Hog-cholera XII. |
| 12 | Hog-cholera bacillus, Arkansas and Nebraska. | Nothing; *i. e.*, control. | 12,800 | 12,800 | 1,600 | 25,600+ | 12,800 |
| 12 | " " | Swine-typhus I | 200 | 200− | 200− | 20,000 | 5,000+ |
| 12 | " " | " II | 200 | 200 | 200− | 20,000 | 20,000 |
| 12 | " " | Hog-cholera XII | | | | 500 | 200− |
| 2 | Swine-typhus I | Nothing; *i. e.*, control. | | 6,400+ | 6,400+ | 6,400+ | |
| 2 | Swine-typhus I | Hog-cholera Arkansas. | | 6,400+ | 6,400+ | 100 | |

the cross-agglutination between the hog-cholera bacilli and these swine paratyphoids, they can readily be distinguished by the type of clumps formed and by absorption tests.

### *Relation to Paratyphoid β from Man.*

As these strains as far as they have been studied culturally are identical with what Jordan (4) and Krumwiede, Kohn, and Valentine (5) regard as typical paratyphoid β, it is of interest to compare serologically the cultures from man with those from swine. Four paratyphoid β cultures which had been isolated comparatively recently were obtained from Dr. Krumwiede, and when tested it

TABLE VI.

*Agglutination of Paratyphoid β Bacilli by Swine-Typhus Sera.*

| Culture tested. | Limit of agglutination in serum of. | | | |
|---|---|---|---|---|
| | Rabbit 13, immune to Swine-typhus I. | | Rabbit 6, immune to Swine-typhus III. | |
| | Limit of agglutination. | Type of sediment. | Limit of agglutination. | Type of sediment. |
| Swine-typhus I | 25,600 | Fluffy. | | |
| " III | 25,600 | " | 51,200 | Fluffy. |
| Paratyphoid β 225 | 6,400 | Compact. | 1,600 | Compact. |
| " β 232 | 12,800 | " | 1,600 | " |
| " β 234 | 6,400 | " | 1,600 | " |
| " β 246 | 12,800 | " | 800 | " |

was found that they did not agglutinate to the titer limit with the serum of rabbits immune to the swine-typhus cultures. This is shown in Table VI. The numbers of the paratyphoid cultures are the ones used by Krumwiede, Kohn, and Valentine (5).

Not only do these human cultures fail to agglutinate to the titer limit of the sera but the clumps when first formed are small and granular and after standing in the refrigerator over night the sediment is compact, resembling that formed by non-motile bacteria.

Rabbits were at once immunized against two of the human paratyphoid strains and the swine cultures tested, with the results given in Table VII. It will be seen that the swine cultures fail to agglu-

TABLE VII.

*Agglutination of Swine-Typhus Bacilli by Paratyphoid β Sera.*

| Culture tested. | Limit of agglutination in serum of. | | | |
|---|---|---|---|---|
| | Rabbit 14, immune to Paratyphoid β 225. | | Rabbit 15, immune to Paratyphoid β 246. | |
| | Limit of agglutination. | Type of sediment. | Limit of agglutination. | Type of sediment. |
| Paratyphoid β 225 | 25,600 | Fluffy. | 25,600 | Fluffy. |
| " β 246 | 25,600 | " | 25,600 | " |
| Swine-typhus I | 6,400 | Compact. | 25,600 | Compact. |
| " II | 6,400 | " | 25,600 | " |
| " III | 6,400 | " | 25,600 | " |
| " IV | 6,400 | " | 25,600 | " |
| " V | 3,200 | " | 6,400 | " |

TABLE VIII.

*Absorption of Agglutinins from Swine-Typhus and Paratyphoid β Sera by Paratyphoid β and Swine-Typhus Bacilli Respectively.*

| Serum. | | | Cultures tested with highest dilution in which agglutination occurred. | | | |
|---|---|---|---|---|---|---|
| Rabbit No. | Immune to. | Absorbed with. | Swine-typhus I. | Swine-typhus III. | Paratyphoid β 232. | Paratyphoid β 246. |
| 13 | Swine-typhus I | Nothing; *i. e.*, control. | 25,600 | 25,600 | 12,800 | 12,800 |
| 13 | " I | Paratyphoid β 232 | 25,600 | 25,600 | 200— | 200— |
| 13 | " I | Paratyphoid β 246 | 25,600 | 25,600 | 200— | 200— |
| | | | Swine-typhus I. | Swine-typhus III. | Paratyphoid β 225. | Paratyphoid β 246. |
| 14 | Paratyphoid β 225 | Nothing; *i. e.*, control. | 6,400 | 6,400 | 25,600 | 25,600 |
| 14 | " β 225 | Swine-typhus I | 200— | 200— | 12,800 | 12,800 |
| | | | Swine-typhus II. | Swine-typhus IV. | Paratyphoid β 225. | Paratyphoid β 246. |
| 15 | Paratyphoid β 246 | Nothing; *i. e.*, control. | 25,600 | 25,600 | 25,600 | 25,600 |
| 15 | " β 246 | Swine-typhus II | 200— | 200— | 6,400 | 12,800 |
| 15 | " β 246 | " IV | 200 | 200 | 12,800 | 12,800 |

tinate to the titer limit in one serum, while in the other they do agglutinate to the titer limit, but in both sera the sediment is compact, while the sediment formed by the human culture is fluffy, resembling a mass of loose cotton.

Absorption tests, the results of which are given in Table VIII, show that the swine-typhus cultures will not absorb the agglutinins from the human paratyphoid $\beta$ sera, nor will the paratyphoid $\beta$ bacilli absorb the agglutinins from the swine-typhus serum.

TABLE IX.

*History of Cultures Which Resemble Swine-Typhus Cultures in Their Agglutination.*

| Culture tested. | History. | | | | | Agglutinated by swine-typhus serum, titer 51,200. |
|---|---|---|---|---|---|---|
| | Isolated by. | Date. | Locality. | Source. | Diagnosis. | |
| Paracolon VI | Dr. N. B. Wherry. | 1908 | San Francisco, Cal. | Liver of 3 yr. boy. | Status lymphaticus. | 25,600 |
| Mouse-typhus I | Dr. Marshal Fabyan. | 1911 | Mass. | Mouse. | Mouse typhoid. | 51,200 |
| Guinea pig-typhus I | Dr. Theobald Smith. | 1895 | " | Guinea pig. | Pseudotuberculosis. | 51,200 |
| Guinea pig-typhus IV | Dr. P. A. Lewis. | 1908 | " | " " | | 51,200 |
| Cattle-typhus. | Dr. Mohler. | 1904 | Washington, D. C. (?). | Cow's brain. | Suspected rabies. | 25,600 |
| Pigeon-typhus. | " " | 1904 | New Jersey. | Heart's blood of pigeon. | Infectious enteritis. | 25,600 |

We have seen that absorption tests show that the hog-cholera bacilli and paratyphoid $\beta$ differ from these swine-typhus cultures, and the question arises as to whether the last are distinctly different from the paratyphoids that are so widely distributed among various animals. With this question in mind the series of stock cultures collected by Dr. Smith was tested against one of the anti-swine-typhus sera and six cultures were found that agglutinated to the titer limit of the serum and that gave the characteristic fluffy clumps formed by the immunizing strain. It is interesting to note that the cultures from the boy, pigeon, and cow had been sent in as possible

hog-cholera bacilli on account of their unusual virulence for laboratory animals. Culturally they correspond to paratyphoid $\beta$ or to the swine-typhus cultures and not to the hog-cholera bacillus. The source of these cultures and the date of their isolation are given in Table IX.

It will be seen that the cultures have come from a variety of animals and from widely different localities in the United States. They not only form characteristic clumping in the serum of rabbits immune to the swine-typhus cultures but they absorb the agglutinins for the immunizing culture as is shown in Table X. The

TABLE X.

*Absorption of Agglutinins from Swine-Typhus Serum by Paratyphoids from Other Species.*

| Swine-typhus serum. | |
|---|---|
| Absorbed with. | Limit of agglutination for immunizing strain. |
| Nothing; *i. e.*, control | 51,200 |
| Paracolon VI | 2,500 |
| Mouse-typhus I | 2,500 |
| Guinea pig-typhus I | 800 |
| " " IV | 500 |
| Cow-typhus | 400 |
| Pigeon-typhus | 3,200 |

absorption has not been complete in any case but this is probably due to the facts that the serum was of very high titer and not enough bacteria were added. The reduction is, however, great enough to show that an absorption has taken place.

### *Complement Fixation Experiments.*

Another method of studying the relationship of such closely allied groups of organisms is by means of the complement fixation test. Antigens were prepared from two paratyphoid $\beta$ strains, two swine-typhus strains, and two hog-cholera bacilli cultures as follows:

The growth from one Blake bottle agar slant was suspended in 10 cc. of sterile distilled water and transferred to a sterile bottle

that was tightly closed. The bottles were heated 1 hour in a water bath at 60°C. and then shaken ½ hour, after which they were placed in the refrigerator for 7 days. They were again shaken for ½ hour

TABLE XI.

*Complement Fixation Tests Using Swine-Typhus Antigens.*

| 0.05 cc. of serum. | | Readings and amounts of antigen. | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Swine-typhus I. | | | Swine-typhus III. | | | |
| Rabbit No. | Immune to. | 0.005 cc. | 0.0025 cc. | 0.00125 cc. | 0.01 cc. | 0.005 cc. | 0.0025 cc. | 0.00125 cc. |
| 13 | Swine-typhus I | 0 | ++ | ++++ | 0 | 0 | 0 | ++++ |
| 16 | " III | 0 | ++ | C. | 0 | 0 | ++ | ++++ |
| 15 | Paratyphoid β 246 | 0 | ++ | " | 0 | 0 | ++ | C. |
| 14 | " β 225 | 0 | +++ | " | 0 | 0 | +++ | " |
| 12 | Hog-cholera Arkansas and Nebraska. | +++ | C. | " | C. | C. | C. | " |
| 17 | Hog-cholera XII | C. | " | " | " | " | " | " |
| 18 | *B. pullorum* III | " | " | " | " | " | " | " |
| 19 | Normal. | " | " | " | " | " | " | " |

TABLE XII.

*Complement Fixation Tests Using Paratyphoid β Antigens.*

| 0.05 cc. of serum. | | Readings and amounts of antigen. | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Paratyphoid β 225. | | | Paratyphoid β 246. | | | |
| Rabbit No. | Immune to. | 0.005 cc. | 0.0025 cc. | 0.00125 cc. | 0.01 cc. | 0.005 cc. | 0.0025 cc. | 0.00125 cc. |
| 13 | Swine-typhus I | 0 | 0 | +++ | 0 | 0 | 0 | 0 |
| 16 | " III | 0 | 0 | ++ | 0 | 0 | 0 | 0 |
| 15 | Paratyphoid β 246 | 0 | 0 | +++ | 0 | 0 | 0 | ++ |
| 14 | " β 225 | 0 | 0 | +++ | 0 | 0 | 0 | ++ |
| 12 | Hog-cholera Arkansas and Nebraska. | +++ | C. | C. | 0 | C. | C. | C. |
| 17 | Hog-cholera XII | C. | " | " | ++++ | " | " | " |
| 18 | *B. pullorum* III | " | " | " | ++ | " | " | " |
| 19 | Normal. | " | " | " | C. | " | " | " |

and placed in the refrigerator for 7 days. At the end of this time 9 cc. of each suspension were added to 1 cc. of 8 per cent sodium chloride solution in a tube and centrifugalized at high speed

for 1 hour. These gave slightly opaque solutions that, on account of their high complement-fixing properties, had to be diluted before they could be used. The hemolytic system used was anti-sheep corpuscle rabbit serum, washed sheep corpuscles, and guinea pig

TABLE XIII.

*Complement Fixation Tests Using Hog-Cholera Bacillus Antigens.*

| 0.05 cc. of serum. | | Readings and amounts of antigen. | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Hog-cholera XII. | | | | Hog-cholera Arkansas. | | | |
| Rabbit No. | Immune to. | 0.01 cc. | 0.005 cc. | 0.0025 cc. | 0.00125 cc. | 0.01 cc. | 0.005 cc. | 0.0025 cc. | 0.00125 cc. |
| 13 | Swine-typhus I | 0 | ++++ | C. | C. | ++ | C. | C. | C. |
| 16 | Swine-typhus III | C. | C. | " | " | +++ | " | " | " |
| 15 | Paraty-phoid *β* 246 | 0 | C. | C. | C. | 0 | ++++ | C. | C. |
| 14 | Paraty-phoid *β* 225 | ++++ | " | " | " | +++ | C. | " | " |
| 12 | Hog-cholera Arkansas and Nebraska. | 0 | + | ++++ | C. | ++ | +++ | C. | C. |
| 17 | Hog-cholera XII | 0 | 0 | 0 | ++++ | 0 | ++ | ++++ | " |
| 18 | *B. pullorum* III | C. | C. | C. | C. | C. | C. | C. | C. |
| 19 | Normal. | " | " | " | " | " | " | " | " |

complement. The hemolytic amboceptor, complement, and antigens were titrated and all the controls made, but only the actual results of the test are given in Tables XI to XIII. The method used was to decrease the amounts of antigen and use a constant amount of serum. The readings given are those of hemolysis, 0 indicating no

hemolysis or complete fixation of complement, ++ slight, +++ moderate, ++++ almost complete, and C. complete hemolysis.

The results show that the swine-typhus and paratyphoid β antigens act in practically the same manner, causing fixation to occur to about the same degree with sera of animals immune to either group, and causing little or no cross-fixation with sera of rabbits immune to the hog-cholera bacillus. The hog-cholera bacilli antigens are more irregular in their action, for they cause some fixation when used with the sera of rabbits immune to either the swine-typhus or the paratyphoid β cultures, but fixation with hog-cholera sera takes place with higher dilution of the antigens.

In the light of the agglutination tests complement fixation does not give the marked differentiation of the strains that one would expect, but on the contrary, the close interrelation of the members of this group is clearly shown.

*Cross-Immunization.*

All the above tests show that the five swine-typhus cultures are more closely related to paratyphoid β than they are to the hog-cholera bacillus. It was surprising, therefore, to find that rabbits immune to these swine-typhus cultures were also immune to a virulent hog-cholera bacillus. Animals immune to each of the cultures have been tested and only one, inoculated with Swine-typhus V, died as the result of the injection of hog-cholera bacilli.

The immunity is one of degree only, for while the animals are apparently little affected by the hog-cholera bacilli and live for months, they show upon autopsy a much enlarged spleen, and when large bits of this organ are transferred to bouillon a pure culture of hog-cholera bacillus is obtained. A similar condition in rabbits inoculated with hog-cholera bacilli of low virulence has been described by Dr. Smith (12).

The question naturally arises as to whether paratyphoid β will immunize rabbits to the hog-cholera bacillus, but it will be seen by examining the results given in Table XIV that in two instances the rabbits inoculated with paratyphoid β were not immune, whereas animals treated in the same way with swine-typhus cultures showed

little or no reaction to an inoculation with virulent hog-cholera bacilli. From the results of this experiment it seems that these swine-typhus cultures are more closely related to the hog-cholera bacillus than are the paratyphoid β cultures.

TABLE XIV.

*Immunity Tests.*

| Rabbit No. | Immunization. | | Aug. 16, 1918. Subcutaneous injection 1 millionth cc. of 24 hr. bouillon culture of Hog-cholera XII. No. of organisms injected 1,810. | |
|---|---|---|---|---|
| | Culture used. | Inoculations of bouillon cultures. | Weight. | Result. |
| | | *1918* | *gm.* | |
| 14 | Paratyphoid β 225 | July 5. 0.01 cc. subcutaneously. " 22. 0.01 " intravenously. | 2,026 | Death in 10 days. Typical lesions of hog-cholera bacillus infection. |
| 15 | Paratyphoid β 246 | " 5. 0.01 " subcutaneously. " 25. 0.01 " intravenously. | 1,822 | Death in 11 days. Typical findings at autopsy. |
| 13 | Swine-typhus I | " 5. 0.01 " subcutaneously. " 25. 0.01 " intravenously. | 1,533 | Pyrexia, but no loss in weight. |
| 16 | Swine-typhus III | " 22. 0.01 " subcutaneously. " 31. 0.01 " intravenously. | 1,917 | No pyrexia or loss in weight. |
| 20 | *B. bronchisepticus.* | " 17. 0.2 " " | 1,412 | Death in 10 days. Typical lesions. |
| 21 | Control. | | 1,848 | Injection one-tenth that given the others. Death in 11 days. Typical lesions. |

## SUMMARY AND CONCLUSIONS.

During the course of some experimental work on hog-cholera, paratyphoid bacilli were isolated from 16 per cent of the pigs. Culturally these organisms are the same as paratyphoid β isolated from man, while they show several differences from hog-cholera bacilli. In their slight pathogenic effect on rabbits they also differ from the hog-cholera bacillus. In their agglutination in sera produced by the injection of living cultures, one of the cultures, isolated from a chronic case, corresponds to *Bacillus enteritidis*, while the other five are apparently in a class by themselves. They resemble paratyphoid β

more closely than hog-cholera bacilli, but the type of clumps formed and absorption experiments show that they are different from either. Whether these differences are enough to make it necessary to put them into a class by themselves is questionable, but the fact that when injected into rabbits they produce an immunity to the hog-cholera bacillus, while paratyphoid $\beta$ does not, is additional evidence in favor of such a classification. Complement fixation experiments have been of little value in differentiating the members of this group, but on the contrary show their close relationship. It seems probable that some of the cultures that are described in the literature as hog-cholera bacilli really belong to this group, which would account for much of the confusion that exists in the classification of the interesting, truly pathogenic bacillus that at one time was thought to be the cause of hog-cholera and in the series of animals with which we have worked has not appeared once. Whether the ingestion of pork containing these bacilli would cause disease in man is a question that can only be decided by a more careful bacteriological study of the organisms causing food poisonings and paratyphoid fever.

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# STANDARDIZATION OF ANTIMENINGOCOCCIC SERUM.

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The establishment of antimeningococcic serum as a specific therapeutic agent for epidemic or meningococcus meningitis antedated Dopter's[1] discovery of the duality of meningococci. Previous to Dopter's studies the effort to produce a so called polyvalent antimeningococcic serum was wholly empirical. Several or many different strains of meningococcus were employed for inoculation on the supposition that such biological variations as occurred in the species would be thus covered. The discovery by Dopter of the parameningococcus necessitated a change of procedure. The strains used for inoculation of horses were now chosen according to whether they conformed serologically to the so called normal or regular meningococcus or the parameningococcus. Wollstein,[2] who studied two of Dopter's cultures as well as a considerable number of strains isolated in the United States, confirmed not only the serological distinctions between normal and parameningococci, but made the important observation that between these two extreme types a number of intermediate varieties were intercalated. These intermediates inclined either toward the normal or the para organisms but were distinguishable serologically from both.

The studies of the meningococcus stimulated by the appearance of epidemic meningitis in the armies of all the belligerent powers have served to confirm and in some degree to extend the observations of Dopter and of Wollstein. The present point of view is represented

[1] Dopter, C., *Compt. rend. Soc. biol.*, 1909, lxvii, 74.

[2] Wollstein, M., *J. Exp. Med.*, 1914, xx, 201.

by the classifications of Gordon[3] and of Nicolle and his associates.[4] Briefly stated they distinguish also two main types of meningococci designated either Types I and II or A and B. Gordon also recognizes Subtypes III and IV, one affiliating with Type II and the other with Type I, and Nicolle equally distinguishes two such types, C and D, which show similar affiliations.

The serological subdivision of the meningococcus into varieties or types has undoubtedly marked a forward step in our knowledge of the causation and specific treatment of epidemic meningitis. It may, however, perhaps be regarded as of questionable value to set up too many minor varieties or types. The conditions are not dissimilar to those existing among the bacilli of dysentery with which we have long been familiar. In both instances the bacterial groups—meningococci and dysentery bacilli—seem still to be in a state of specific flux. In the case of meningococcus this fact is made especially apparent by the study of carrier strains which show even greater serological fluctuation than do the strains obtained from the inflamed meninges.[5]

However, the present chief interest centers around the establishment of a test of therapeutic efficiency in antimeningococcic serum. It does not yet appear feasible to treat epidemic meningitis on the basis of monovalent sera adapted in every instance to the particular type or variety of meningococcus inducing the infection. Nor does it seem necessary to attempt this. There are no insuperable difficulties in the way of preparing an adequate polyvalent antimeningococcic serum in which not only the two main kinds of antibodies are sufficiently represented but also the main subvarieties or types as well. Meningococci do not exhibit such strict serological specificity as we have become familiar with in regard to the types of pneumococci.[6] On the contrary, there is much overlapping of antibody

[3] Gordon, M. H., *Great Britain National Health Insurance, Med. Research Com., Special Rep. Series, No. 3*, 1917, 10.

[4] Nicolle, M., Debains, E., and Jouan, C., *Ann. Inst. Pasteur*, 1918, xxxii, 150.

[5] Eastwood, A., *Rep. Local Gov. Bd. Pub. Health and Med. Subjects*, 1917, N.S. cxiv, 1. Griffith, F., *ibid.*, 52. Scott, W. M., *ibid.*, 111. (See also *J. Hyg.*, 1918, xvii, 63, 124, 191.) Gordon, M. H., *J. Hyg.*, 1918, xvii, 290.

[6] Dochez, A. R., and Gillespie, L. J., *J. Am. Med. Assn.*, 1913, lxi, 727.

reactions, a fact which affects undoubtedly the wide therapeutic applicability of the antimeningococcic serum.

In spite of this overlapping it is imperative that a standard should be established which will afford a measure of the therapeutic efficacy of given samples of the serum. An efficient sample of the serum should contain definite amounts of antibodies for the principal varieties and the main subvarieties or types of the meningococcus. There will be no disagreement as to the two main types, while differences of opinion may arise as to the main subvarieties. As regards the latter it will be safe to follow Gordon's classification which appears to express the essential fact. Accordingly the polyvalent antimeningococcic serum should be prepared with at least four properly chosen cultures of the meningococcus. This is the point of view arrived at by the New York State Department of Health, which has adopted and is enforcing in the State a standard based on agglutination titer.[7]

In choosing a standard of value for the antimeningococcic serum certain questions at once arise. The first relates to the particular antibody on which therapeutic activity depends. The point at issue with an antibacterial serum, as represented by the antimeningococcic serum, is far more complex than with the ordinary antitoxic sera. According to present beliefs the antimeningococcic serum acts (*a*) by increasing phagocytosis of the meningococcus,[8] (*b*) by neutralizing endotoxin,[9] (*c*) by injuring directly the meningococci and impairing their power of propagation.[10] It remains, therefore, to be determined whether any one criterion will suffice as a measure of these several forms of activity.

The methods employed to effect the standardization of the serum have from time to time undergone change. Thus there have been employed successively (*a*) opsonin content,[11] (*b*) complement-fixing

[7] *Off. Bull., New York State Dept. Health*, 1918, iii, No. 5.

[8] Jochmann, *Deutsch. med. Woch.*, 1911, xxxvii, 1733. Flexner, S., *J. Exp. Med.*, 1907, ix, 168. Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1916, xxiii, 683.

[9] Flexner, S., *J. Exp. Med.*, 1907, ix, 168.

[10] Flexner, *J. Exp. Med.*, 1907, ix, 105. Flexner, S., and Jobling, J. W., *J. Exp. Med.*, 1908, x, 141.

[11] Jobling, J. W., *J. Exp. Med.*, 1909, xi, 614.

titer,[12] (*c*) agglutinin content,[13] (*d*) antiendotoxic value,[14] and (*e*) protective power.[15]

The discrimination of parameningococcus from the regular meningococcus has automatically rendered the opsonic and complement-binding methods obsolete, since they do not suffice for the separate determination of the discrete antibodies. In practice, the discussion of standards has narrowed itself down to the relative merits of agglutinins or protective power as a measure of value.

It is desirable to state here that agglutination is the method of choice for standardization, since it alone gives clear distinctions between the types and varieties of meningococci. Its employment has been questioned on the ground that as the therapeutic action of the serum is probably not a function of the agglutinin, antibody may not be a measure of therapeutic efficacy. An effort is being made, therefore, to substitute for the agglutinin content of the serum the protective power.

In endeavoring to set up a standard of protection, account must, in the first instance, be taken of the small and variable pathogenic action of the meningococci. This fact has indeed been generally recognized by investigators. While certain small animals, such as young guinea pigs weighing about 100 gm.[16] and mice, will often succumb to intraperitoneal inoculations of moderate doses of cultures of meningococci, yet their reaction is variable, and fluctuation in virulence of the cultures is common.

## EXPERIMENTAL.

Recently, therefore, Hitchens and Robinson[15] have proposed a method of standardization of the antimeningococcic serum based on its protective power. Because of the intrinsic importance of the subject for our military organizations and our civil communities, we have submitted the method to a critical examination.

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[12] Krumbein and Schatiloff, P., *Deutsch. med. Woch.*, 1908, xxiv, 1002.

[13] Amoss, H. L., and Wollstein, M., *J. Exp. Med.*, 1916, xxiii, 403. Amoss, H. L., *J. Am. Med. Assn.*, 1917, lxix, 1137.

[14] Flexner, *J. Exp. Med.*, 1907, ix, 168. Dopter, C., *Compt. rend. Soc. biol.*, 1909, lxvi, 772. Kraus, R., and Doerr, R., *Wien. klin. Woch.*, 1908, xxi, 12. Gordon, M. H., *Brit. Med. J.*, 1918, i, 110.

[15] Hitchens, A. P., and Robinson, G. H., *J. Immunol.*, 1916, i, 345.

[16] Flexner, *J. Exp. Med.*, 1907, ix, 168.

Hitchens and Robinson give in their paper what we regard as illustrative protocols of their experiments, from which they conclude that the "protection test parallels the extent of immunization more nearly than agglutination or complement fixation tests." They also state that "there seems to be a considerable degree of specificity in the test." A close study of their protocols fails, we believe, to carry conviction that, even in their hands, the test is either delicate or decisive. Unless it is both delicate and decisive, the object which they seek, namely that "if the amount of serum necessary to protect against one M. L. D. of culture is considered as a unit, a rational and uniform method of standardizing antimeningococcus serum can be obtained," and "a dosage similar to that of antitoxic serum would be possible by such a method of standardization" is not attained.

In carrying out our experiments we have followed closely the conditions laid down by Hitchens and Robinson. Thus a 16 hour growth of the meningococcus chosen was suspended in 1 cc. of dilute guinea pig serum and injected immediately in the indicated doses into the peritoneal cavity of white mice. In testing the action of samples of the antimeningococcic serum, 0.5 cc. was injected intraperitoneally 2 hours before the suspension of the culture was inoculated also intraperitoneally. Finally, in instances in which several cultures of a given meningococcus were made, they were all washed off with the dilute guinea pig serum and pooled before being used for inoculation. The volumes of serum and of bacterial suspension employed have always been 0.5 cc. each.

### *Series I. Regular Meningococcus.*

A regular meningococcus having a suitable degree of virulence was selected. The suspension was prepared in the manner indicated and injected immediately into the peritoneal cavity (Table I). As Table I shows, all but one of the injected mice died; but the order of their death did not conform to the dosage of the culture. When death took place within 24 hours the peritoneal cavity and heart's blood yielded many or innumerable colonies of meningococcus; when death was delayed the number of colonies grown from the heart's blood was often few.

The next experiment was made with normal serum as a control for the antimeningococcic serum, and in duplicate in order to cover in a measure the variable response of the mice to the inoculation (Table

TABLE I.

*Virulence Test of Regular Meningococcus 4.*

| Weight of mice. | Dose of suspension. | Result. |
|---|---|---|
| *gm.* | *cc.* | |
| 17 | 0.5 | D.* 13 hrs. |
| 15.5 | 0.25 | " 43 " |
| 15 | 0.12 | " 17 " |
| 14 | 0.06 | S. R. |
| 14 | 0.03 | D. 9 hrs. |

* In the tables D. indicates died, S.R., sick and recovered, R., recovered.

II). The protocol brings out the facts (1) that the reaction of the mice to the inoculation is irregular, and (2) that normal horse serum exhibits no protective power against the meningococcus. Meningococci in large numbers were cultivated from the peritoneal cavity and heart's blood of the mice dying within 24 hours.

TABLE II.

*Normal Horse Serum + Regular Meningococcus 4.*

| Weight of mice. | | Dose of serum. | Dose of suspension. | Result. | |
|---|---|---|---|---|---|
| Lot A. | Lot B. | | | Lot A. | Lot B. |
| *gm.* | *gm.* | *cc.* | *cc.* | | |
| 16 | 16 | 0.5 | 0.5 | D. 14 hrs. | D. 9 hrs. |
| 16 | 17 | 0.5 | 0.25 | " 13 " | S. R. |
| 15 | 15 | 0.5 | 0.12 | " 9 " | D. 57 hrs. |
| 14 | 15 | 0.5 | 0.06 | " 37 " | R. |
| 13 | 14 | 0.5 | 0.03 | " 9 " | D. 85 hrs. |

Two experiments were made with polyvalent antimeningococcic serum of high titer according to the standard based on agglutinin content (Tables III and IV). The serum also proved highly effective in the treatment of cases of epidemic meningitis. While Table III might be interpreted as giving evidence of protection, Table IV indicates an irregularity merely in the reaction of the mice, which cannot depend on protection. All the mice of Lot A which died yielded innumerable colonies of meningococcus from the peritoneal cavity and heart's blood.

TABLE III.

*Antimeningococcic Serum + Regular Meningococcus 4.*

| Weight of mice. | Dose of serum. | Dose of suspension. | Result |
|---|---|---|---|
| *gm.* | *cc.* | *cc.* | |
| 18 | 0.5 | 0.5 | D. 15 hrs. |
| 17.5 | 0.5 | 0.25 | " 15 " |
| 16.5 | 0.5 | 0.12 | S. R. |
| 17 | 0.5 | 0.06 | " |
| 17 | 0.5 | 0.03 | D. 9 hrs. |

TABLE IV.

*Antimeningococcic Serum + Regular Meningococcus 4.*

| Weight of mice. | | Dose of serum. | Dose of suspension. | Result. | |
|---|---|---|---|---|---|
| Lot A. | Lot B. | | | Lot A. | Lot B. |
| *gm.* | *gm.* | *cc.* | *cc.* | | |
| 15 | 17 | 0.5 | 0.5 | D. 29½ hrs. | S. R. |
| 16 | 18 | 0.5 | 0.25 | " 21 " | " |
| 16 | 15 | 0.5 | 0.12 | " 24 " | D. 24 hrs. |
| 14 | 15 | 0.5 | 0.06 | " 29 " | S. R. |
| 12 | 12 | 0.5 | 0.03 | S. R. | " |

The next tests were made with a parameningococcus and the same samples of normal and antimeningococcic horse serum.

*Series II. Parameningococcus.*

The chief point which the experiment brings out is the striking irregularity of the protection tests in this instance (Tables V to VII).

TABLE V.

*Control, Parameningococcus G.*

| Weight of mice. | Dose of suspension. | Result. |
|---|---|---|
| *gm.* | *cc.* | |
| 20 | 0.5 | D. 27½ hrs. |
| 16 | 0.25 | " 25½ " |
| 14 | 0.12 | " 35 " |
| 14 | 0.06 | " 24 " |
| 15 | 0.03 | " 3½ "* |

* Excluded; probable accident.

TABLE VI.

*Normal Horse Serum + Parameningococcus G.*

| Weight of mice. | Dose of serum. | Dose of suspension. | Result. |
|---|---|---|---|
| *gm.* | *cc.* | *cc.* | |
| 16 | 0.5 | 0.5 | D. 16 hrs. |
| 15 | 0.5 | 0.25 | S. R. |
| 15 | 0.5 | 0.12 | D. 29½ hrs. |
| 15 | 0.5 | 0.06 | " 16 " |

TABLE VII.

*Antimeningococcic Serum + Parameningococcus G.*

| Weight of mice. | | Dose of serum. | Dose of suspension. | Result. | |
|---|---|---|---|---|---|
| Lot A. | Lot B. | | | Lot A. | Lot B. |
| *gm.* | *gm.* | *cc.* | *cc.* | | |
| 17 | 16 | 0.5 | 0.5 | D. 23 hrs. | S. R. |
| 16 | 15 | 0.5 | 0.25 | S. R. | " |
| 16 | 16 | 0.5 | 0.12 | D. 6½ hrs. | D. 10½ hrs. |
| 15 | 14 | 0.5 | 0.06 | S. R. | S. R. |
| 14 | 14 | 0.5 | 0.03 | D. 17 hrs. | " |

## *Series III. Intermediate Meningococcus.*

The next test was carried out with an intermediate or irregular meningococcus tending slightly toward the regular (Tables VIII and IX). According to the agglutinin standard the polyvalent serum employed in this experiment was efficient; according to the protection test it is irregular in action and of practically no potency.

TABLE VIII.

*Control, Intermediate Meningococcus 30.*

| Weight of mice. | Dose of suspension. | Result. |
|---|---|---|
| *gm.* | *cc.* | |
| 15 | 0.5 | D. 10½ hrs. |
| 14 | 0.25 | " 10½ " |
| 14 | 0.12 | S. R. |
| 14 | 0.06 | D. 34 hrs. |
| 13 | 0.03 | R. |

TABLE IX.

*Antimeningococcic Serum + Intermediate Meningococcus 30.*

| Weight of mice. | | Dose of serum. | Dose of suspension. | Result. | |
|---|---|---|---|---|---|
| Lot A. | Lot B. | | | Lot A. | Lot B. |
| *gm.* | *gm.* | *cc.* | *cc.* | | |
| 15 | 14 | 0.5 | 0.5 | D. 8 hrs. | D. 10 hrs. |
| 13 | 13 | 0.5 | 0.25 | " 8 " | " 16 " |
| 14 | 12 | 0.5 | 0.12 | " 10 " | " 27½ " |
| 13 | 13 | 0.5 | 0.06 | " 8 " | " 16 " |
| 12 | 12 | 0.5 | 0.03 | " 29 " | S. R. |

The final tests were carried out with two samples of monovalent antimeningococcic serum prepared in the horse, one for the normal or regular and the other for the parameningococcus. The horses yielding the two samples of serum had been under immunization for 12 and 9 weeks respectively. The agglutination titers of the sera at the time the protection tests were made was + to ++ at 1:500 dilution. According to the standard followed at The Rockefeller Institute the horses were not yet ready to yield therapeutic sera. The object was to ascertain whether they would show any evidence of protective power.

### *Series IV. Monovalent Sera.*

In Tables X and XI Lots 1 and 4 refer to the number of the regular culture of meningococcus used for inoculation. According to Table XI the monovalent serum would have to be regarded as devoid of antibodies, which is obviously, in view of the agglutination titer, not the case.

Perhaps it would have been well to carry the suspensions to a greater dilution; namely, to doses of 0.06 and 0.03 cc. Since these tests were performed during the period last spring of acute mouse shortage, the smaller doses were omitted, especially as in the control tests the mice receiving 0.06 cc. both survived. The fact that of those given the monovalent serum one survived and the other died merely emphasizes the irregularity of this test.

TABLE X.

*Control, Regular Meningococci 1 and 4.*

| Weight of mice. | | Dose of suspension. | Result. | |
|---|---|---|---|---|
| Lot 1. | Lot 4. | | Lot 1. | Lot 4. |
| *gm.* | *gm.* | *cc.* | | |
| 15 | 15 | 0.5 | D. 30½ hrs. | D. 28 hrs. |
| 15 | 15 | 0.25 | " 28 " | " 36 " |
| 15 | 15 | 0.12 | S. R. | " 30 " |
| 14 | 14 | 0.06 | " | S. R. |

TABLE XI.

*Regular Monovalent Serum + Regular Meningococci 1 and 4.*

| Weight of mice. | | Dose of serum. | Dose of suspension. | Result. | |
|---|---|---|---|---|---|
| Lot 1. | Lot 4. | | | Lot 1. | Lot 4. |
| *gm.* | *gm.* | *cc.* | *cc.* | | |
| 15 | 17 | 0.5 | 0.5 | D. 17½ hrs. | D. 11 hrs. |
| 13 | 15 | 0.5 | 0.25 | " 18 " | " 15½ " |
| 13 | 14 | 0.5 | 0.12 | S. R. | " 13 " |

Tests with monovalent para serum and parameningococci were also carried out. Three parameningococcus cultures were employed (Tables XII and XIII). It is obvious from Tables XII and XIII that the tests give no indication of protective value in the serum in

TABLE XII.

*Control, Parameningococci 60, 79, and 85.*

| Weight of mice. | | | Dose of suspension. | Result. | | |
|---|---|---|---|---|---|---|
| Lot 60. | Lot 79. | Lot 85. | | Lot 60. | Lot 79. | Lot 85. |
| *gm.* | *gm.* | *gm.* | *cc.* | | | |
| 15 | 15 | 15 | 0.5 | D. 9½ hrs. | D. 37 hrs. | D. 2½ hrs.* |
| 15 | 15 | 15 | 0.25 | " 31 " | " 37 " | " 33 " |
| 14 | 14 | 14 | 0.12 | " 31 " | " 31 " | " 3 " * |
| 14 | 14 | 14 | 0.06 | S. R. | " 33 " | " 31 " |

* Excluded; accident.

TABLE XIII.

*Monovalent Parameningococcic Serum + Parameningococci 60, 79, and 85.*

| Weight of mice. | | | Dose of serum. | Dose of suspension. | Result. | | |
|---|---|---|---|---|---|---|---|
| Lot 60. | Lot 79. | Lot 85. | | | Lot 60. | Lot 79. | Lot 85. |
| *gm.* | *gm.* | *gm.* | *cc.* | *cc.* | | | |
| 15 | 17 | 17 | 0.5 | 0.5 | D. 11 hrs. | D. 11 hrs. | D. 19 hrs. |
| 15 | 15 | 15 | 0.5 | 0.25 | " 7 " | S. R. | " 11 " |
| 15 | 14 | 16 | 0.5 | 0.12 | S. R. | D. 13 hrs. | " 19 " |
| 13 | 13 | 13 | 0.5 | 0.06 | " | " 21 " | " 20 " |

spite of a moderate content of agglutinin and doubtless of therapeutic principles.

The last two series of experiments were reversed so that the monovalent sera were made to react with their opposite cultures—the regular serum with para cultures and the para serum with regular cultures. Aside from what appeared to be an accidental survival, all the inoculated mice succumbed.

## DISCUSSION.

In carrying out the experiments described in this paper no attempt was made to restudy, in an intensive manner, the general subject of the protective power of the antimeningococcic serum on animals. The purpose was merely to repeat the experiments of Hitchens and Robinson on which they base their argument for a protection standard for the antimeningococcic serum.

Our results failed wholly to confirm those of Hitchens and Robinson and conform to the earlier studies in indicating that infection tests carried out in laboratory animals with the meningococcus give extremely variable results, and that this factor of variability enters into and affects unfavorably the protection tests.

Investigators are generally agreed that the antimeningococcic serum possesses protective power against experimental meningococcic infection. This power is shown either by the survival of the inoculated and serum-treated animal or by reduction in the number of meningococci and increase in intensity of the phagocytosis within the peritoneal cavity. But not only are these reactions irregular

in their occurrence but they are confined within relatively narrow limits and may succeed only when the minimum lethal dose of the culture inoculated is not exceeded. Reactions of this order are not well suited for purposes of standardization.

## CONCLUSIONS.

Experiments were made for the purpose of testing the reaction of protection against infection as a measure of potency of antimeningococcic serum.

The results of the experiments were extremely variable and bore no relation to the quality of the sera as determined by the period of immunization of the horses from which they were obtained, or the indications of efficiency based upon their employment in human cases of epidemic meningitis.

The results also failed entirely to conform to the agglutination titer of the sera tested and to be affected by the different type forms of the meningococci.

We regard the protective power for laboratory animals of the antimeningococcic serum as an unsuitable index of its value in human medicine and as inferior to the agglutination titer as a standard of potency.

# A STUDY OF THE CHANGES IN VIRULENCE OF THE PNEUMOCOCCUS AT DIFFERENT PERIODS OF GROWTH AND UNDER DIFFERENT CONDITIONS OF CULTIVATION IN MEDIA.*

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Considered broadly, there are two fundamental factors which determine the pathogenic activities of bacteria, growth in the tissues of the host and injury of the tissues locally or systemically. It is not clear in the different infectious processes to how great an extent one phase,—growth or simple multiplication, or pure parasitism—is dependent upon the other phase,—injury resulting from the production of toxic substances. Students of infection and immunity, however, are now drawing each year more precise distinctions between the parasitic and the toxicogenic activities of the agents of infection. Thus in leprosy and tuberculosis on the one hand, the parasitism of the agents is generally recognized to be dominant, while in diphtheria and tetanus, on the other hand, the parasitism has long been known to be extremely limited and largely, if not wholly overshadowed by the action of the powerful toxins which the organisms produce locally. In other infections, notably the bacteriemic infections and especially pneumococcus infection, the full significance of each of these factors is at present indeterminate chiefly on account of our lack of knowledge of the conditions affecting not only the toxicogenic activities of the agents during infection but their parasitism as well.

* Read in abstract at the meeting of the American Society of Pathologists and Bacteriologists, New York, April, 1917.

Our conception of parasitism connotes adaptation—lacking the necessary adaptation, an organism could not become parasitic. Attention has thus been directed to this fundamental and essential quality which the pathogenic bacteria possess and which distinguishes them from all others. As a result of numerous observations on the effect of animal passage upon bacteria, the virulence, or degree of pathogenicity, has become so intimately associated with one factor in the adaptation of the organism, the medium, that another equally important factor, the growth energy, has hitherto received scant attention. It is the purpose of the following paper to present the results of a preliminary study of this essential relation between the different phases of growth and the degree of virulence of the agent.

Comparatively few observations on increase of virulence produced outside the body tissues are recorded and these are found chiefly in the studies of the early investigators. Thus according to Roger,[1] while the vegetative characteristics and the virulence of the streptococcus diminish progressively during repeated transfers in broth, both the vegetative power and the pathogenic action are again enhanced when serial transplants of the attenuated strain are made in normal rabbit serum. He considered that the action of serum outside the animal organism was therefore almost identical with that of the tissues of the organism. Von Lingelsheim,[2] also working with the streptococcus, was able to confirm these results only within certain limits. He was never able by successive serum transfers to increase the virulence of *Streptococcus pyogenes*, for example, so that a single injection was fatal for mice. Von Lingelsheim found, however, that the virulence was in most instances increased more rapidly when diminishing minimal doses were used and when the transfers were made from animal to animal. In our experience there has occurred sudden and marked decrease of virulence of pneumococcus strains recovered from dead rabbits kept for an interval of several days or weeks at a low temperature. The maintenance of virulence by preservation of pneumococci in dried spleens of mice, as first suggested by Heim,[3] has, however, been used extensively.

While Eyre and Washbourn[4] also reported increase of virulence by animal passage and decrease by prolonged artificial cultivation, they as well as others

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[1] Roger, G. H., *Compt. rend. Soc. biol.*, 1890, xlii, 573.

[2] von Lingelsheim, W., *Z. Hyg. u. Infectionskrankh.*, 1891, x, 331; 1892, xii, 308; *Beitr. exp. Therap.*, Heft I, 1899, 1, 49.

[3] Heim, L., *Z. Hyg. u. Infectionskrankh.*, 1905, l, 123.

[4] Eyre, J. W., and Washbourn, J. W., *J. Path. and Bacteriol.*, 1897, iv, 394; *Lancet*, 1899, i, 19.

made further observations on the marked variations that occur in the increase of virulence of different strains of the pneumococcus isolated from cases of lobar pneumonia and from normal persons. In these investigations, however, repeated animal passage was used for increasing the virulence.

Cotoni[5] studied the virulence of the pneumococcus in culture and in the animal body. He stated that by using gelatin and special peptone media containing no body fluids he was able to raise the virulence for mice of certain originally avirulent strains. Fluctuations in virulence of strains carried on *in vitro* were also noted.

Hilbert[6] found that association of *B. diphtheriæ* and streptococcus in culture tended to produce a more rapid and increased production of toxin, a result which he considered due chiefly to changes in the medium occurring as a result of the growth of the streptococcus. Thus streptococcus culture filtrate, he stated, was an excellent medium for toxin production. Attempts to raise the virulence of *B. diphtheriæ* by cultivation with streptococci or other organisms have, however, generally proved unsuccessful. Williams[7] cultivated two avirulent strains with virulent streptococci for 90 generations without any change in virulence. Roger[1] reported that he had been able to make avirulent pneumococci and streptococci virulent by injecting one or more drops of a sterile culture or extract of *B. prodigiosus* into rabbits at the same time. Although non-pathogenic, *prodigiosus* cultures are nevertheless toxic and thus might act as a predisposing agent favoring the development of streptococcus infection. Roger apparently did not test the effect of combining in culture prior to injection.

The latent period of growth of bacteria after transfer to a fresh medium and the diminution in growth due to inhibition after the maximum has been reached, have long been recognized. Müller[8] called attention to variations in the length of the latent period or lag depending upon the age of the parent culture. Barber[9] first found that the latent period might be avoided if an actively dividing organism accustomed to the medium was used for inoculation. Penfold[10] studied the nature of bacterial lag and Ledingham and Penfold[11] have contributed a mathematical analysis of the lag phase in the growth of bacteria, in which they showed that the generation time decreased steadily and uniformly until the minimal length or commencement of the second or logarithmic phase was reached. Chesney[12] has recently made investigations of these phases of bacterial growth under

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[5] Cotoni, L., Thèse de Paris, 1912, No. 78.

[6] Hilbert, P., *Z. Hyg. u. Infectionskrankh.*, 1898, xxix, 157.

[7] Williams, A. W., *J. Med. Research*, 1902, viii, 83.

[8] Müller, M., *Z. Hyg. u. Infectionskrankh.*, 1895, xx, 245.

[9] Barber, M. A., *J. Infect. Dis.*, 1908, v, 379.

[10] Penfold, W. J., *J. Hyg.*, 1914, xiv, 215.

[11] Ledingham, J. C. G., and Penfold, W. J., *J. Hyg.*, 1914, xiv, 242.

[12] Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.

different conditions and at different stages of the culture. But the possible relation between these phases of growth and the virulence of the organism has apparently never been studied.

The present study of changes in virulence of the pneumococcus in culture, under different conditions of growth, was undertaken not only for the purpose of obtaining a clearer conception of the immediate factors involved but also in the hope that, as a result of a more precise knowledge of the conditions of culture at different stages of the growth of the pneumococcus, it might be possible to develop better methods for producing immune serum of greater potency. The strain selected for the tests was a standard Type I pneumococcus, which had been maintained at a uniformly high degree of virulence for a number of years by means of frequent animal passage. When the interval between passages exceeded 2 days, the strain was stored in a special semisolid serum medium, in which it had been found that a constant level of virulence could be held for several weeks.[13] Previous tests had, however, shown that a rapid decline of virulence was likely to occur unless the above methods were rigidly observed.

For purposes of comparison the experiments included repeated transfers at 8 and 24 hour intervals of subcultures from the standard strain both in its original virulent and in its later attenuated relatively non-virulent state. The 8 hour transfers were made in order that the latent period and also the period of inhibition of growth might be avoided. The organisms were grown in 1 per cent peptone meat infusion broth prepared by neutralizing to 0.2 per cent acid (phenolphthalein) before heating and coagulating the meat proteins, and also in the same medium to which 5 per cent citrated rabbit blood had been added. One set of the blood cultures was grown in a thin layer of the medium exposed to the air while in the other a surface layer of albolene excluded the oxygen of the air and gave fairly anaerobic conditions of growth after the oxygen absorbed by the corpuscles had been taken up by the pneumococcus.

While it was realized that parallel bacterial counts and acidity and hydrogen ion estimations at different periods of growth in con-

[13] Wadsworth, A., *Proc. New York Path. Soc.*, 1903, iii, 113.

nection with the virulence tests would have been of considerable value, the few tests that it was possible to carry out proved insufficient to allow of definite conclusions.

*Reduction of Virulence during Cultivation by Repeated Transfers at Intervals of 24 Hours.*

Two series of transfers at 24 hour periods were made in meat infusion broth (Table I). In the first series, undertaken originally for another purpose, only one virulence test was made up to the 17th week when 1 cc. of the 24 hour growth, injected intraperitoneally, failed to kill a mouse. Tested at intervals up to the 32nd week, mice receiving 1 cc. of the culture, with one or two exceptions, not only survived but failed to show any abnormal symptoms.

In the second series virulence tests were made at the end of the 2nd, 3rd, 6th, and 8th weeks, when the transfers were discontinued. In 3 weeks 0.000001 cc. did not kill in less than 46 hours; on the contrary a mouse receiving 0.1 cc. survived. By the 6th week still more complete loss of virulence had occurred, 1 cc. of the 24 hour growth failing to kill.[14] Thus, it was shown that, except in rare instances in which slightly greater susceptibility in the individual mouse or irregularities in cultural growth might be important factors, marked reduction in virulence had been established.[15]

[14] Marked differences in the power to retain virulence in artificial medium are shown by different strains of the pneumococcus. In our experience a Type I strain cultivated in plain meat infusion broth medium and transferred at 24 hour intervals for 14 months has killed in 42 hours, when 0.000001 cc. of the 24 hour growth is inoculated into mice. Transferred at 48 hour intervals for nearly 1 year 0.000001 cc. has even proved fatal in approximately 2 days. A standard Type II and a Type III strain also required repeated transfers for many weeks before a marked decrease in virulence was obtained.

[15] Later tests of the attenuated Type I culture during continued transfers in broth for more than a year have shown at times evidence of slight fluctuations, 1 cc. for one or two successive tests proving fatal to mice. These fluctuations appear, however, to be infrequent and transitory.

TABLE I.

*Decrease in Virulence by Repeated Transfers in Broth at 24 Hour Intervals. Results of Virulence Tests on Mice Inoculated with 24 Hour Growth.*

| Medium and series. | Parent culture 18 hr. growth. | Transferred every 24 hrs. for. | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2 wks. | 3 wks. | 4 wks. | 6 wks. | 8 wks. | 17 wks. | 18 wks. | 20 wks. | 21 wks. | 23 wks. | 24 wks. | 30 wks. | 30½ wks. | 32 wks. |
| Plain broth medium; 1st series. | 0.000001 cc. D.*—46 hrs. | | | 0.01 cc. D. 41 hrs. | | | 1 cc. L. | 1 cc. L. | 1 cc. L. 1 " " | 1 cc.D. 54 hrs. | 1 cc. L. 1 " " | 1 cc. L. 0.6 " " | 1 cc.D. 48 hrs. | 1 cc. L. 1 " " | 1 cc. L. 1 " D. 30 hrs. |
| Plain broth medium; 2nd series. | 0.000001 cc. D. — 46 hrs. | 0.001 cc. L. | 0.1 cc. L. | | 1 cc. L. | 1 cc. L. 1 " " | Discontinued. | | | | | | | | |

* In the tables D. indicates died, L., lived.

*Maintenance of Virulence during Rapid Cultivation by Repeated Transfers at Intervals of 8 Hours.*

Cultures of the standard virulent strain after recent passage through mice were used in all the series of these experiments. Three series of tests were made in plain broth and in the two blood media previously described (Table II). In the first series after 2 weeks, or 42 transfers, virulence was maintained; after 12 weeks, or 252 transfers, mice receiving 0.000001 cc. succumbed after a slightly increased interval. In the second series virulence held in both blood cultures up to the 5th week, when owing to contaminations they were discontinued. The third series was continued until the 13th week, or for 273 transfers. The organisms grown in the plain broth medium maintained their virulence unimpaired. Some fluctuations occurred in the blood cultures, although, with one exception, all mice inoculated with 0.000001 cc. succumbed. These slight irregularities, occurring in this and other tests in which the medium contained 5 per cent blood, were probably due in part to the medium, as at times separation of the blood elements and the broth occurred, affecting the growth of the culture.

The results of this experiment stand in striking contrast to those of the previous experiment. In one the virulence of the pneumococcus was maintained for 13 weeks with little or no loss; in the other virulence decreased rapidly and in 6 weeks was practically lost. In one the pneumococcus was under rapid cultivation; in the other the pneumococcus was cultivated more slowly. In the first experiment and in one series of the second experiment the same plain broth medium was used, to which had been added no special enriching material. It was thus shown that the presence of blood, serum, or other tissue fluid is not essential and that the virulence of the pneumococcus may be maintained by rapid cultivation alone. This is of special significance and suggested further experiments to determine the effect of rapid cultivation on cultures which had been attenuated and were practically avirulent.

TABLE II.

*Maintenance of Virulence by Repeated Transfers in Broth at 8 Hour Intervals. Results of Virulence Tests on Mice Inoculated with 8 Hour Growth.*

| Medium and series. | Transferred every 8 hrs. for. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2 wks. | 4 wks. | 5 wks. | 6 wks. | 7 wks. | 9 wks. | 11 wks. | 12 wks. | 13 wks. |
| Plain broth medium; 1st and 2nd series. | 1st series: 0.000001 cc. D. 38 hrs. | | 2nd series: not tested, growth too slight. | | | | | 1st series: 0.000001 cc. D. 50 hrs. | |
| Plain broth medium; 3rd series. | | 0.0001 cc. D.—29 hrs. 0.000001 cc. D. 100 hrs. | 0.000001 cc. D.—53 hrs. | 0.000001 cc. D. 32 hrs. | 0.000001 cc. D. 42 hrs. | 0.000001 cc. D.—38 hrs. | 0.000001 cc. D. 42 hrs. | | 0.000001 cc. D. 32 hrs. |
| Plain broth + citrated 5 per cent normal rabbit blood; aerobic; 1st and 2nd series. | 1st series: 0.000001 cc. D.—31 hrs. | | 2nd series: 0.000001 cc. D. 30 hrs. | | | | | 1st series: 0.000001 cc. D.—63 hrs. | |
| The same medium; 3rd series. | | 0.000001 cc. D. 30 hrs. | | 0.000001 cc. D. 40 hrs. | 0.000001 cc. D. 95 hrs. | 0.000001 cc. D.—38 hrs. | 0.000001 cc. D. 42 hrs. | | 0.000001 cc. D. 54 hrs. |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Plain broth + citrated 5 per cent normal rabbit blood; anaerobic; 1st and 2nd series. | 1st series: 0.000001 cc. D. 23 hrs. | | 2nd series: 0.000001 cc. D. 33 hrs. | | | | | 1st series: 0.000001 cc. D. 50 hrs. | |
| The same medium; 3rd series. | | 0.000001 cc. D. 44 hrs. | | 0.000001 cc. D. 40 hrs. | 0.000001 cc. D. 40 hrs. | 0.000001 cc. D.—38 hrs. | 0.000001 cc. D.—42 hrs. 0.000001 cc. D.—47 hrs. | 0.000001 cc.* D. 5 days, 6 hrs. | 0.00001 cc.* D. 40 hrs. 0.000001 cc.* L. |

* Growth slight and unsatisfactory, owing to separation of blood elements and partial clotting.

TABLE III.

*Increase in Virulence by Repeated Transfers in Broth at 8 Hour Intervals. Results of Virulence Tests on Mice Inoculated with 8 Hour Growth.*

| Medium and series. | Parent culture 24 hr. growth. | 3 transfers. | 9 transfers. | 21 transfers. | | | | |
|---|---|---|---|---|---|---|---|---|
| Plain broth medium; 1st series. | 1 cc. plain broth. L. | 1 cc. L. | 1 cc. D.—36 hrs. | 1 cc. L.<br>0.5 " "<br>0.01 " " | | | | |
| | **Parent culture 24 hr. growth.** | **3 transfers.** | **9 transfers.** | **18 transfers.** | **30 transfers.** | **42 transfers.** | **63 transfers.** | **84 transfers.** |
| Plain broth medium; 2nd series. | 1 cc. plain broth. L. | 1 cc. D. 45 hrs.<br>1 " L. | 1 cc. D.—31 hrs.<br>1 " " —40 "<br>0.5 " " 48 " | 1 cc. D.—42 hrs.<br>0.5 " " 50 "<br>0.1 " " —42 "<br>0.1 " " —54 "<br>0.01 " " —56 " | 0.1 cc. D. 5 days, 9 hrs.<br>0.01 cc. D. 41 hrs.<br>0.001 " L.<br>0.001 " " | 1 cc. D. 44 hrs.<br>0.5 " " 33 "<br>0.5 " " —42 "<br>0.1 " " —66 " | 1 cc. D. 30 hrs.<br>0.5 " L.<br>0.1 " "<br>0.01 " D. 71 " | 1 cc. D.—88 hrs.<br>1 " " 5 days, 17 hrs.<br>0.5 cc. D. 7 days, 10 hrs.<br>0.5 cc. L.<br>0.1 " "<br>0.1 " " |
| | **Parent culture 24 hr. growth.** | **3 transfers.** | **9 transfers.** | **21 transfers.** | | | | |
| Plain broth + citrated 5 per cent normal rabbit blood; aerobic; 1st series. | 1 cc. plain broth. L. | 1 cc. D.—32 hrs. | 1 cc. D. 26 hrs. | Not tested. | | | | |

| | Parent culture 24 hr. growth. | 3 transfers. | 9 transfers. | 18 transfers. | 24 transfers. | 36 transfers. | 78 transfers. | |
|---|---|---|---|---|---|---|---|---|
| The same medium; 2nd series. | 1 cc. plain broth. L. | 1 cc. D. 25 hrs.<br>1 " " —49 " | 1 cc. D.—31 hrs.<br>1 " " 42 "<br>0.5 " " 4 days, 5 hrs. | 1 cc. D. 8 days, 5 hrs.<br>0.5 cc. D. 6 days, 18 hrs.<br>0.1 cc. L.<br>0.1 " "<br>0.01 " " | 1 cc. D. 30 hrs.<br>0.5 " L.<br>0.1 " "<br>0.1 " " | 1 cc. D. 33 hrs.*<br>0.5 " L. | 1 cc. D.—24 hrs.<br>1 " " —40 "<br>0.5 " " —48 "<br>0.5 " L. | |

| | Parent culture 24 hr. growth. | 3 transfers. | 9 transfers. | 21 transfers. | | | | |
|---|---|---|---|---|---|---|---|---|
| Plain broth + citrated 5 per cent normal rabbit blood; anaerobic; 1st series. | 1 cc. plain broth. L. | 1 cc. D.—32 hrs. | 1 cc. D. 72 hrs. | 1 cc. D. 31 hrs.<br>0.5 " L.<br>0.1 " D.—41 hrs. | | | | |

| | Parent culture 24 hr. growth. | 3 transfers. | 9 transfers. | 18 transfers. | 30 transfers. | 42 transfers. | 84 transfers. | |
|---|---|---|---|---|---|---|---|---|
| The same medium; 2nd series. | 1 cc. plain broth. L. | 1 cc. D.—41 hrs.<br>1 " " 31 "<br>0.5 " L. | 1 cc. D. 48 hrs.<br>1 " " —5 days, 6 hrs.<br>0.5 cc. D.—40 hrs. | 1 cc. D. 36 hrs.<br>0.5 " L.<br>0.1 " "<br>0.1 " D. 8 days, 18 hrs. | 1 cc. D. 19 hrs.<br>0.5 " L.<br>0.1 " "<br>0.1 " " | 0.1 cc. D.—42 hrs.<br>0.5 " L. | 1 cc. D. 18 hrs.<br>1 " L.<br>0.5 " D. 47 hrs.<br>0.5 " L. | |

These results indicate fluctuations in virulence of the 8 hour growth possibly due to slight changes in the technique of making the transfers, in the medium, or in the inoculation of the cultures. It is difficult to maintain uniform conditions throughout 84 transfers, especially when blood medium is used. The results of such slight changes would naturally be more evident in the 8 hour than in the 18 or 24 hour growth. In each table the complete experiment is recorded in order to show these variations. Obviously, whenever the method of transferring the culture fails to maintain the vegetative power, the virulence also will fail.

* Staphylococcus contamination. Culture continued from preceding 8 hour transfer.

*Increase of Virulence during Rapid Cultivation by Repeated Transfers at Intervals of 8 Hours.*

The effect of rapid transfers on the culture which had lost its virulence after repeated seedings at 24 hour intervals in plain broth was studied. 1 cc. of this culture for some time had proved to be innocuous for mice. Seedings of a 24 hour growth were made in its own broth and in the two blood media (Table III). In the preliminary series the virulence was tested at the end of the 1st, 3rd, and 7th days. After three 8 hour transfers mice receiving 1 cc. of the blood cultures died in less than 32 hours. Control mice receiving 1 cc. of uninoculated blood medium were apparently unaffected by the inoculation. After nine transfers each mouse, including that inoculated with the culture in plain broth, succumbed. 1 cc. of the anaerobic blood was fatal after twenty-one seedings, but the same amount of the plain broth culture failed to kill. The aerobic culture in 5 per cent blood broth was not tested. While these results were incomplete and showed considerable irregularity, they were considered of sufficient significance to warrant repetition.

In the second series transfers were made at 8 hour intervals and the virulence was tested from time to time for approximately 4 weeks. The plain broth and anaerobic blood cultures were transferred 84 times; the aerobic culture in blood 78 times. After three 8 hour seedings in media with or without blood five out of six mice inoculated with 1 cc. died in less than 49 hours; after nine transfers five died in less than 48 hours, the sixth after several days. 0.5 cc. was also fatal. In all subsequent tests, with one exception, 1 cc. of the 8 hour growth killed, though in a few instances death was considerably delayed. In these as in the other tests recorded pneumococci were invariably obtained from the heart's blood at autopsy. While the cultures containing blood showed more immediate return of virulence, the increase in virulence was on the whole most marked in the plain broth cultures, 0.5, 0.1, and even 0.01 cc. at times causing death. Thus, without animal passage the virulence of the attenuated culture, 1 cc. of which had previously failed to kill, was increased by rapid transfers in plain broth at 8 hour intervals, so that inoculation of 0.1 and 0.01 cc. was fatal.

TABLE IV.

*Fluctuations in Virulence of Attenuated Culture during the 24 Hour Period of Growth.*

| Medium and test. | Parent culture 24 hr. growth. | Tested at. | | | |
|---|---|---|---|---|---|
| | | 4 hrs. | 6 hrs. | 8 hrs. | 24 hrs. |
| Plain broth medium; 1st test. | * | A.† 1 cc. L.<br>B. 1 " D. 7 days. | A. 1 cc. D. —36 hrs.<br>B. 1 cc. D. —36 hrs. | A. 1 cc. D. —34 hrs.<br>B. 1 cc. L. | A. 1 cc. L.<br>B. 0.6 " " |
| The same medium; 2nd test. | * | Not tested. | 1 cc. D.—34 hrs.<br>1 cc. D.—18 hrs.<br>0.5 cc. L.<br>0.5 " "<br>0.1 " " | 1 cc. D.—81 hrs.<br>1 cc. L.<br>0.5 " "<br>0.5 " " | * |
| The same medium; 3rd test. | * | Not tested. | 1 cc. D. 52 hrs.<br>1 " " 25 "<br>0.5 " " L.<br>0.5 " " " | Not tested. | * |
| The same medium; 4th test. | 1 cc. L.<br>1 " " | A. 1 cc. L.<br>B. 1 " " | A. 1 cc. D. 18 hrs.<br>B. 1 cc. D. 48 hrs. | A. 1 cc. D. —29 hrs.<br>B. 1 cc. D. 16 hrs. | A. 1 cc. L.<br>B. 1 " " |

*For a period of 6 weeks before and after these experiments control tests of this culture in broth after growth for 24 hours at 37°C. failed to kill mice in doses of 1 cc. with few exceptions; see Table I, tests from the 17th to the 32nd week.

†A, culture seeded from 24 hour growth. B, inoculated from the same culture as A.

As it was thought that the virulence of both the aerobic and anaerobic cultures containing 5 per cent rabbit blood might be further increased if the blood was reduced to $2\frac{1}{2}$ per cent, since the higher percentage might retard growth, a fresh series was commenced. The virulence of the third and ninth 8 hour generations from the avirulent culture was tested, but no marked change in virulence from that recorded in the previous tests resulted. In all 8 hour cultures and especially in those containing blood, growth is

occasionally sufficiently delayed to affect materially the dosage or number of organisms inoculated, with resulting irregularities.

*Fluctuations in Virulence during the 24 Hour Period of Culture.*

In order to ascertain whether the attenuated 24 hour broth culture might not itself pass through a period of increased virulence, coincident with that of maximum growth, mice were inoculated with the culture on the 4th, 6th, and 8th hours after seeding. The results are given in Table IV. The most marked change was shown at the 6 hour period, all six mice inoculated with 1 cc. dying in less than 52 hours, though four others receiving 0.5 cc. survived. One of two receiving 1 cc. of the 4 hour growth died in about 7 days while two of four inoculated with the 8 hour culture also died. Two controls which received the same culture after the usual 24 hour inoculation were not affected. It therefore appeared that an increase in virulence actually occurred, though this was less marked than in the subcultures which had been transferred repeatedly at frequent intervals for a longer period.[16]

## SUMMARY AND CONCLUSIONS.

It has been possible by rapid transfers alone, not only to maintain the virulence for mice of the pneumococcus in artificial media but also to restore a certain degree of virulence to cultures previously rendered non-virulent by less rapid transfers in the same medium. For these results the presence of enriching fluids such as blood or serum is not required. In addition it has been shown that attenuated cultures, which had been repeatedly demonstrated to be avirulent for mice at the 24 hour period of growth, exhibited marked pathogenicity if injected during, or especially at the commencement of the period of maximum growth when the growth energy may be

[16] In a later test of the fluctuations in virulence during the 24 hour period of growth, the mice which had received the culture at the end of the 6 and 8 hour growth periods died promptly of pneumococcus infection, indicating that this culture possessed as much virulence as those transferred repeatedly at short intervals. The medium, however, was not from the same lot as that used in the previous tests.

considered at its height. In these cultures, however, the increase in virulence was usually less than in others transferred repeatedly at frequent intervals.

The significance of these results is not necessarily limited to pneumococcus infections. Other lines of investigation are suggested which may possibly help to clarify certain conceptions of the relation which the different activities of the bacterial cell as an agent of infection bear to one another and to the host in various infectious diseases. Although the pneumococcus may offer an especially striking example for purposes of demonstration by experiment, it is not probable that the close relation between the vegetative power or growth energy of the pneumococcus and its pathogenic power is peculiar to this organism. The vegetative power may depend upon many conditions affecting both the host and the bacterial agent of infection. Different species of bacteria may acquire or develop it in different degrees under different conditions. But it must assuredly form the basis not only of the essentially parasitic but also of the more special toxicogenic activities of the bacteria.

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